# SYNTHESIS AND BIOLOGICAL EVALUATION OF ACCEPTOR SUBSTRATES FOR ALPHA-1,3-FUCOSYLTRANSFERASE

Shona L. Smith

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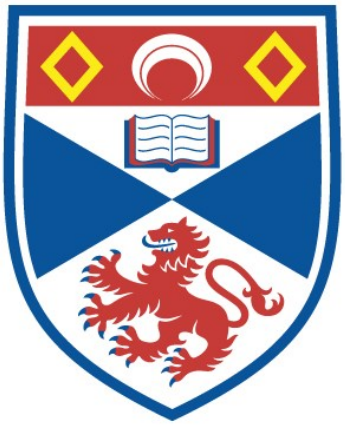
at the
University of St Andrews



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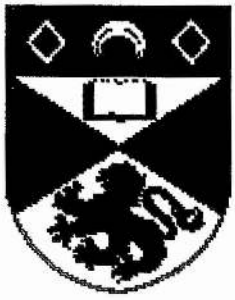
# Synthesis and Biological Evaluation of Acceptor Substrates for α-1,3-Fucosyltransferase



A thesis submitted to the

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for the degree of

DOCTOR OF PHILOSOPHY

By

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October 1997

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## *Abstract*

The chemical synthesis of sulfate and phosphate derivatives of galactose-β-1,4-*N*-acetylglucosamine-*O*R (octyl *N*-acetyllactosamine) and galactose-β-1,3-*N*-acetylglucosamine-*O*R [where R= $-(CH_2)_7CH_3$] are reported here using *N*-acetylglucosamine and galactose as starting materials. Sialylation of octyl *N*-acetyllactosamine derivatives was achieved using *trans*-sialidase. These compounds were evaluated as potential acceptor substrates for five recombinant α-1,3-fucosyltransferases (α-1,3-FucT) and a semi-pure α-1,3/4-FucT (human milk). The kinetic data showed a wide range of acceptor specificities between different recombinant enzymes. Octyl *N*-acetyllactosamine 6-*O*-sulfate proved to be an excellent substrate for α-1,3-FucT VI, with a $K_M$ of 0.85 μM. This substrate has a lower $K_M$ than any reported substrate for any α-1,3-FucT. An unusual result was observed for octyl *N*-acetyllactosamine derivatives containing a sulfate or phosphate group at the site of glycosylation. These compounds were found to be good acceptor substrates for α-1,3-FucT VI and milk α-1,3/4-FucT with $K_M$ and $V_{max}$ values similar to those of the parent compound, octyl *N*-acetyllactosamine. Preliminary studies show that the product of such a reaction could contain a sulfate or phosphate diester linkage between fucose and octyl LacNAc. If the anionic substituent at the site of glycosylation is being fucosylated, current models proposed for a mechanism involving an enzyme active site base mechanism cannot explain this result. An alternative mechanism has been suggested involving $Mn^{2+}$ coordination to the hydroxyl group of the acceptor substrate being glycosylated. This mechanism can also be used to explain the unusual kinetic results obtained for substrates containing a sulfate or phosphate group at the site of glycosylation.

## *Abbreviations*

| | |
|---|---|
| AcOH | acetic acid |
| AgOTf | silver trifluoromethane sulfonate (silver triflate) |
| BzCl | benzoyl chloride |
| collidine | 2,4,6-trimethyl pyridine |
| DMAP | dimethylaminopyridine |
| EDTA | ethylenediaminetetraacetic acid |
| ES-MS | electrospray mass spectroscopy |
| FAB-MS | fast atom bombardement mass spectrometry |
| HEPES | (*N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid]) |
| Hz | frequency in herz |
| I.R. | infra red |
| *m*-CPBA | *meta*-chloroperbenzoic acid |
| m.p. | melting point |
| MALDI | matrix assisted desorption ionisation |
| n.m.r. | nuclear magnetic resonance |
| NIS | *N*-iodosuccinimide |
| *p*-TsOH | *para*-toluenesulfonic acid |
| py | pyridine |
| t.l.c. | thin layer chromatography |
| TFA | trifluoroacetic acid |
| TfOH | trifluoromethanoic acid |
| THF | tetrahydrofuran |
| TMSOTf | trimethylsilyl triflate |
| TOF | time of flight detector |
| TRIS | tris-hydroxymethyl-aminomethane |

## *Carbohydrate Abbreviations*

| | |
|---|---|
| octyl | $-(CH_2)_7CH_3$ |
| LacNAc | Gal-β-1,4-GlcNAc |
| GlcNAc | 2-acetamido-2-deoxy-D-glucopyranose |
| Gal | D-galactose |
| Fuc | L-fucose |
| GDP | guanosine diphosphate |
| UDP | uridine diphosphate |
| $Le^c$ | Gal-β-1,3-GlcNAc |
| $Le^x$ | Gal-β-1,4-(α-1,3-Fuc)-GlcNAc |
| sialyl $Le^x$ | NeuAc-α-2,3-Gal-β-1,4-(α-1,3-Fuc)-GlcNAc |
| α-1,3-FucT | α-1,3-fucosyltransferase |
| β-1,4-GalT | β-1,4-galactosyltransferase |
| NeuAc | sialic acid, 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosyl |

# *Chapter 1: Introduction*

## *1.1 General Background*

It is now widely accepted that carbohydrates are involved in a diverse array of functions in all living systems, in addition to the energy storage (e.g. starch / glycogen) and structural roles (e.g. cellulose / chitin) classically assigned to them. In recent years much interest has focused on glycoconjugates (carbohydrate molecules attached to proteins or lipids) which are involved in mediating 'specific recognition' events and 'modulating' biological processes. Consequently oligosaccharides help to generate the diversity of functions required to develop different cell-types, tissues, organs and species (for review see [1]).

Glycoconjugates are major components of cell surfaces and are often characteristic of cell-type. It is assumed that these cell type-specific saccharides are involved in cellular processes such as antigen binding and serving as ligands in cell-cell interactions [1]. As a result carbohydrates have been found to be important in cell recognition processes, cell growth and cell defense, including roles such as binding of bacterial and viral antigens on mammalian cells, bacterial toxins, blood group determinants and cell-cell recognition processes. Perhaps of more interest is the function of carbohydrates in disease states such as metastasis of cancer, inflammatory responses and bacterial infection. Hence in recent years there has been a growing interest in the development of carbohydrate based drugs within the pharmaceutical industry [2].

Some examples of naturally occurring oligosaccharides and their biological functions are described in the following sections.

## *1.2 Classes of Biologically Active Carbohydrates*

Every organism has a wide variety of glycans which may either be linked to lipids (glycolipids) or proteins (glycoproteins or proteoglycans) or may occur as free carbohydrates.

### 1.2.1 Glycoproteins

Glycoproteins are divided into two main classes depending on their linkage to the protein. *N*-Linked glycoproteins are oligosaccharides which are attached to the side chain of an asparagine residue whereas *O*-linked glycoproteins have carbohydrates attached to the side chain of serine or threonine.

#### *1.2.1.1 N-linked Glycoproteins*

There are three main classes of *N*-glycans, high mannose, complex and hybrid. All are derived from a common trimannosyl core Man-α-1,6-(Man-α-1,3)-Man-β-1,4-GlcNAc-β-1,4-GlcNAc-Asn (**Figure 1.1**). The outer branches differ among the different subtypes (for general information see [3]).

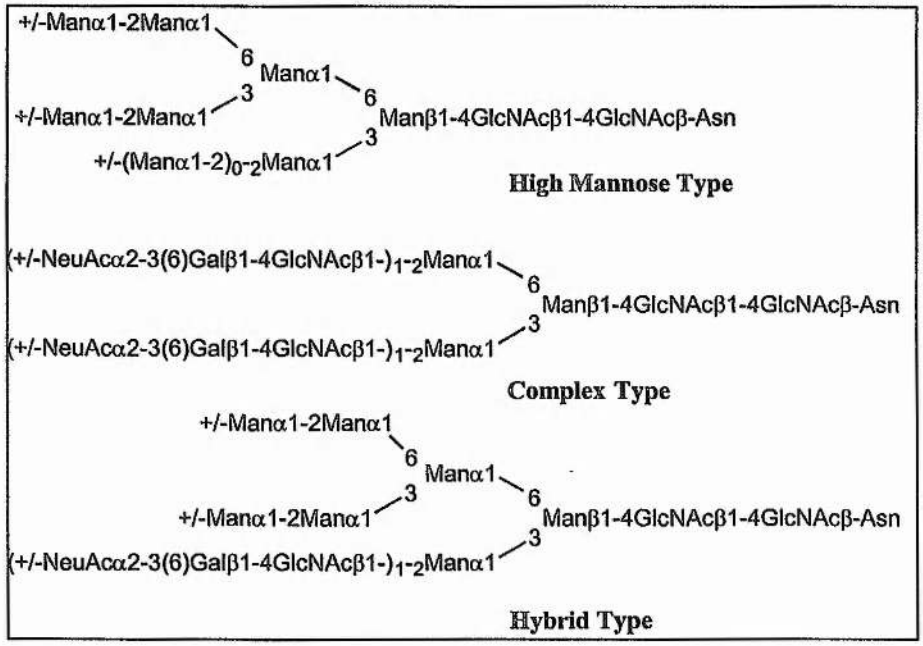


**Figure 1.1: High Mannose, Complex and Hybrid Type *N*-Glycans**

The biosynthesis of the *N*-glycan core is initiated by transfer of a large oligosaccharide, $Glc_3Man_9GlcNAc_2$ from dolichol pyrophosphate to an asparagine residue of a protein. Oligosaccharide processing of these glycans begins with removal of three glucose residues. A series of reactions then proceed until the chain is capped thus forming the three different glycan subtypes. The complex and hybrid type glycans have a terminal unit Gal-β-1,4-GlcNAc which is the core of Lewis x and y antigens. The most common residue capping this sequence is sialic acid (NeuAc) giving sialylated Lewis antigens, for example sialyl Lewis x (**Section 1.3**).

#### *1.2.1.2 O-Linked Glycans*

The *O*-linked glycans have the reducing terminus of an oligosaccharide linked to the hydroxyl side chain of serine or threonine. This class can be further sub-divided into three main groups depending on the type of sugar and amino acid residues involved in the linkage. The first group are the mucin type *O*-glycans which contain an *N*-acetylgalactosamine residue linked to a serine or threonine linkage. The second group also have a sugar linked to a serine or threonine residue but the sugar is *N*-acetylglucosamine (*O*-GlcNAc type).

Unlike *N*-glycans the family of *O*-glycans do not share a common core structure, there are in fact at least four core types (reviewed [3]). Thus the biosynthesis of *O*-glycans is more diverse than that for the corresponding *N*-glycans. The *O*-glycan chain is built up by successive addition of hexose or hexosamine units. Termination of the chain is achieved by sialylation and there are thought to be four different sialyltransferase enzymes involved in this process. An example of an *O*-linked glycan is the glycoprotein GlyCAM-1 (glycosylation-dependent cell-adhesion molecule) which is part of a ligand for a protein L-selectin which is involved in inflammation.

#### *1.2.1.3 Glycosaminoglycans*

The third main class of glycoproteins are the proteoglycans which have a xylose residue attached to a serine or threonine unit. One type of proteoglycan is glycosylaminoglycans (GAGs). There are seven known classes of GAGs (**Figure 1.2**). These are unbranched repeating disaccharide units which are often found covalently attached to proteins forming proteoglycan type structures. The most commonly found

GAGs are chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate and heparan sulfate. Heparin is another highly sulfated GAG which is synthesized as part of a proteoglycan but is more commonly found as free carbohydrate. Keratan sulfate is typically found in connective tissues. The seventh class of GAG, hyaluronan, is synthesized independently of a protein backbone and contains no sulfation. It has the simplest structure containing only repeating disaccharides of glucuronic acid and *N*-acetylglucosamine in alternating β-1,3 and β-1,4 linkages.

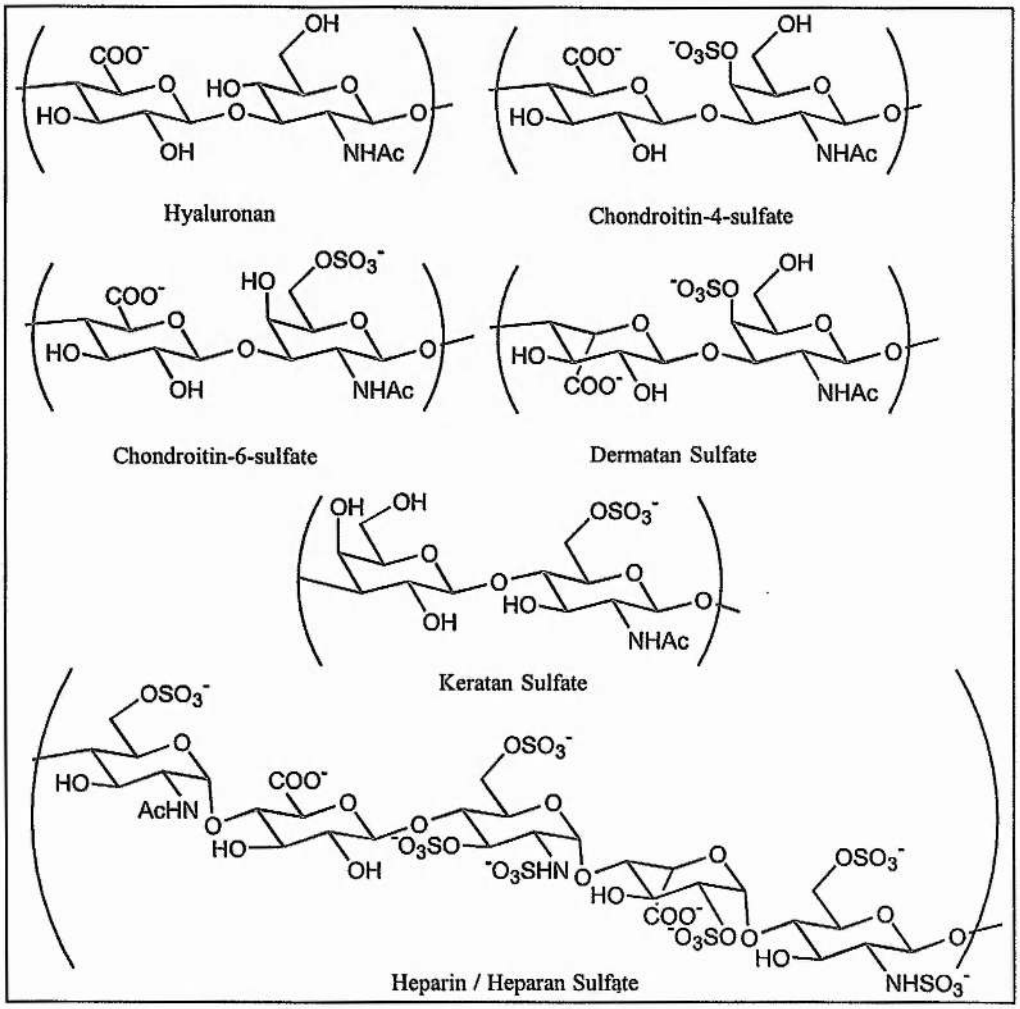


**Figure 1.2: Glycosaminoglycan Structures**

GAGs are known to play an important structural role on the surface of many cell types [4], however, they are also thought to be important in other processes. For example, it

is known that heparin has anticoagulant activity and in fact is used as a prophylactic in the clinical treatment of thrombosis [5].

### 1.2.2 Glycolipids

Carbohydrates linked to lipids embedded in the cell membrane can be divided into two groups. The most commonly found glycolipid, glycosphingolipids, have a ceramide unit attached through a β-linked lactosyl carbohydrate moiety (**Figure 1.3**) (**Section 1.2.2.1**). The second class of glycolipids, glycosyl phosphatidyl inositols (GPI), have a phosphoglycerol unit attached to the carbohydrate chain and form part of a linking arm for the attachment of proteins to membranes (**Section 1.2.2.2**).

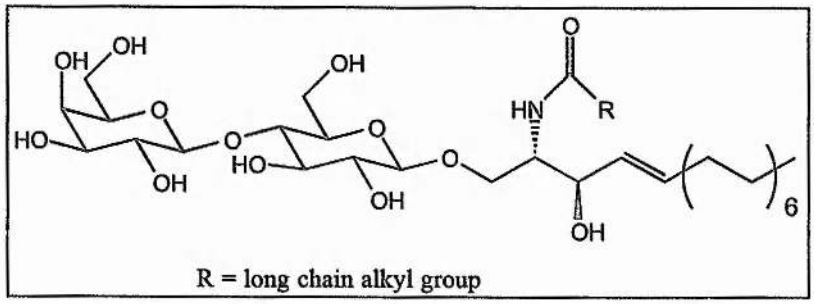


**Figure 1.3: Lactosyl-Ceramide Linkage Found in Glycolipids.**

#### *1.2.2.1 Glycosphingolipids.*

Glycosphingolipids (GSL) are components of eukaryotic cell membranes and can be classified into four major groups according to their core structure (**Table 1-1**) [6].

| | |
|---|---|
| Gangliosides | Gal-β-1,3-GalNAc-β-1,4-Gal-β-1,4-Glc-β-1,1-Cer |
| Globosides | GalNAc-β-1,3-Gal-α-1,4-Gal-β-1,4-Glc-β-1,1-Cer |
| Lactosides (type I) | Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc-β-1,1-Cer |
| Lactosides (type II) | Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc-β-1,1-Cer |

**Table 1-1: Classification of Glycosphingolipids**

Most GSLs contain an acidic monosaccharide group, for example, glucuronic acid, galacturonic acid or sialic acid. In some GSLs the essential core structure may be branched

or some of the sugar residues may contain sulfate groups, for example sulfoglucuronyl glycolipids (SGGL) which are found in peripheral nerves (**Figure 1.4**) [7].

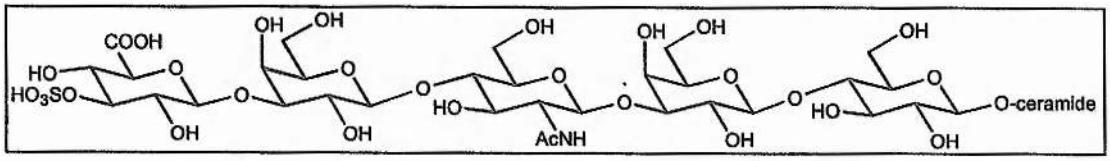


**Figure 1.4: Structure of SGGL Glycolipid.**

### *1.2.2.2 GPI Anchors*

Glycosyl phosphatidyl inositol (GPI) containing glycoproteins are ubiquitous in nature (for review articles see [8], [9]). The oligosaccharide chain is linked to phosphatidyl inositol, which is capable of inserting into the cell - surface lipid bilayer. The non-reducing terminus of the oligosaccharide is linked to the C-terminal carbonyl group of a protein through a phosphoethanolamine unit. The GPI anchor therefore serves as a functional alternative to a membrane spanning peptide domain in the attachment of proteins to the cell surface. The first GPI anchor structure to be determined was that of the variant surface glycoprotein of the African trypanosome, *Trypanosoma brucei* (**Figure 1.5**). This parasite, which causes sleeping sickness, uses its dense GPI - anchored protein coat to evade the immune response of the infected host.

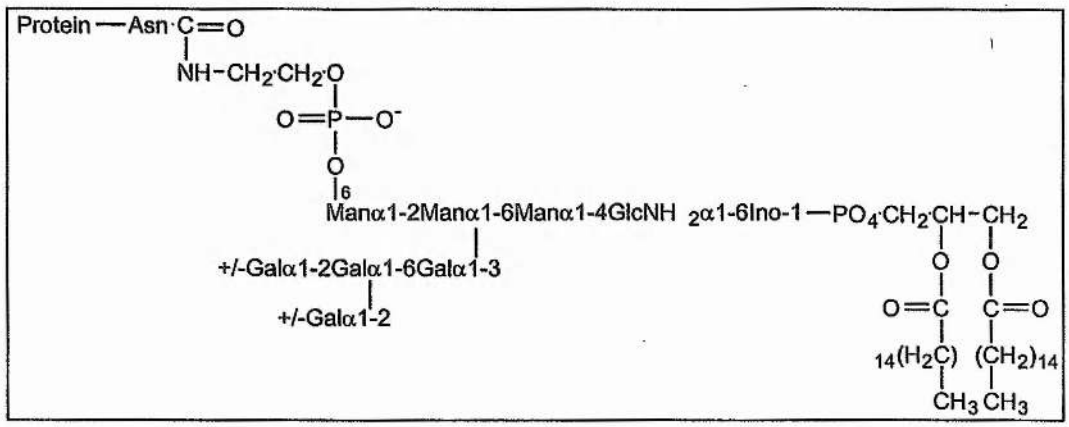


**Figure 1.5: The Structure of the GPI-anchor Variant Surface Glycoprotein Found in *Trypanosoma brucei***

### *1.2.2.3 Lipopolysaccharides*

Lipopolysaccharides (LPS) are released by Gram negative bacteria and are responsible for the toxic effects observed during bacterial infections. The lipid component of LPS's (Lipid A) is embedded in the outer membrane of bacterial cells. Attached to the lipid is a polysaccharide which protrudes into the environment and is composed of two distinct sections. The core section is made up of two sugar rings (commonly *N*-acetylglucosamine residues modified by phosphate groups, **Figure 1.6**) and is attached directly to the lipid component. The second section is a longer chain usually made up of repeating units of three to eight sugars. Linking the core and outer sections are two unusual sugars, a heptose unit (seven carbon sugar) and Kdo (3-deoxy-D-manno-2-octulosonic acid). Kdo is an eight carbon sugar and occurs nowhere else in nature. It is the terminal disaccharide phospholipid (**Figure 1.6**: Lipid A) which has been shown to be responsible for toxic activity. In humans acute inflammatory responses to lipid A results in the release of cytokines and other cellular mediators such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1). It is these cellular mediators which result in the physical disorders associated with infection [10], [11].

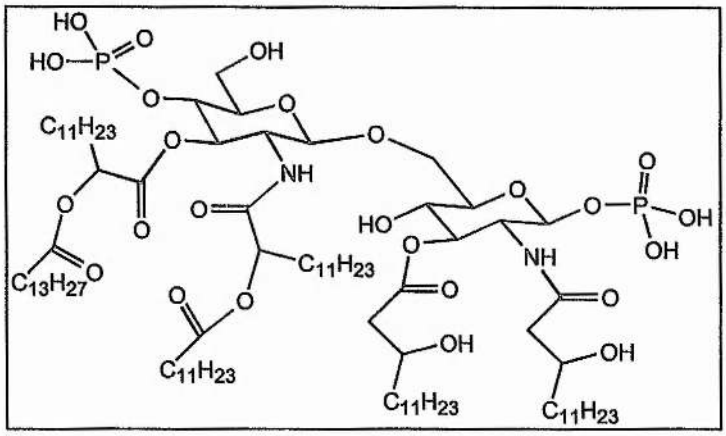


**Figure 1.6: The Lipid A Section From *Escherichia coli***

## *1.3 The Lewis Antigens*

Every organism contains a wide variety of glycoconjugates which may be linked to lipids or *N*-linked or *O*-linked to proteins. These glycoconjugates are typical for the organism and the tissue type. An example of cell-type specific oligosaccharides are the Lewis antigens. These oligosaccharides 'decorate' mammalian cells and can be found attached to lipids or more commonly *N*-linked to proteins. They are involved in many biological processes and have similar structures, biosynthetic pathways and functions in the human body [3]. There are four known Lewis antigens, Lewis a ($Le^a$), Lewis b ($Le^b$), Lewis x ($Le^x$) and Lewis y ($Le^y$) (**Figure 1.7**).

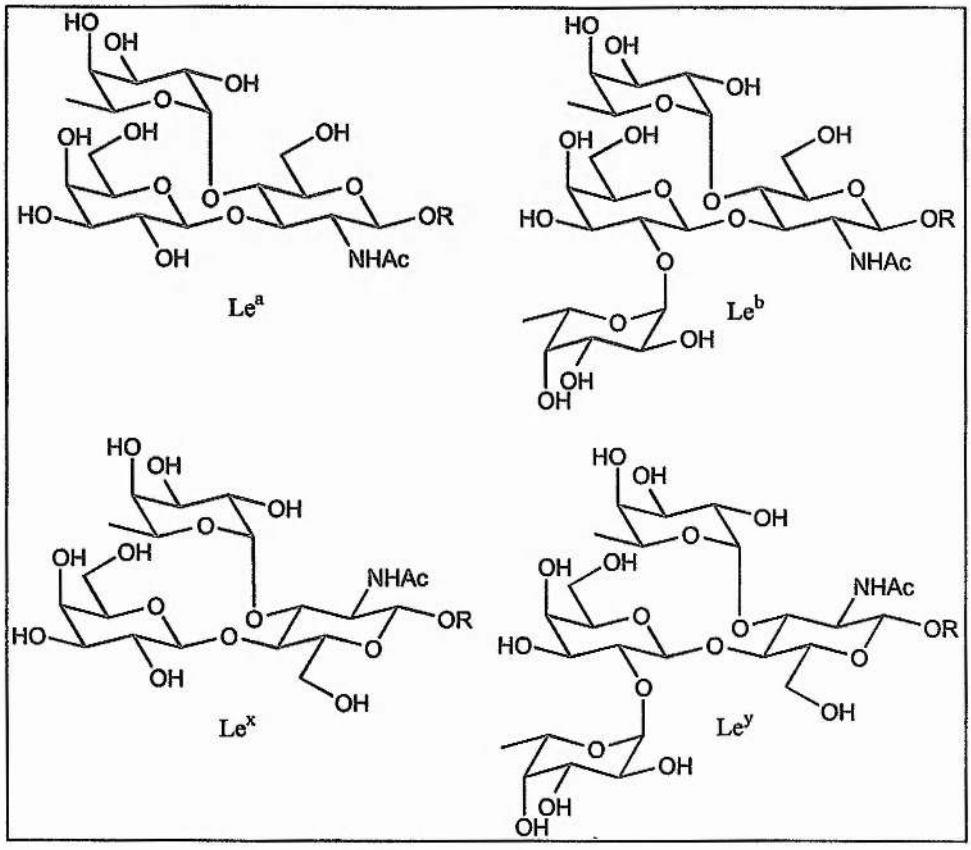


**Figure 1.7: The Lewis a, b, x and y Antigens**

R = glycoprotein or glycolipid

The Lewis a and b oligosaccharides have a type I core derived from Gal-β-1,3-GlcNAc. The core of Lewis antigens x and y are type II chains containing Gal-β-1,4-

GlcNAc (*N*-acetyllactosamine). The core oligosaccharide may be capped with sialic acid residues forming sialylated Lewis antigens, for example sialyl Lewis x (**Figure 1.8**).

### 1.3.1 Biological Role of the Lewis Antigens

Due to the presence of Lewis antigens on the surface of cells, it is thought they may be involved in cell-cell interactions. The diversity of carbohydrate structures found on different cell surfaces may provide specific sites which are recognized by receptor molecules. Evidence to suggest that cell surface carbohydrates do act as ligands for receptors came from the discovery of a group of carbohydrate binding proteins known as lectins. One family of adhesive lectin molecules found in eukaryotic cells are selectins of which there are three types, L (leukocyte)-selectin, P (platelet)-selectin and E (endothelial)-selectin. These all have different functions and are present in different cell types. All the selectins are involved in adhesion between circulating leukocytes and endothelium or platelets (for a general review see [3]). The adhesion of leukocytes is dependent on carbohydrate-protein interactions. All of the Lewis antigens are involved in cell-cell interactions but for the purposes of this thesis it is the role of the antigen sialyl Lewis x (**Figure 1.8**) which is of interest.

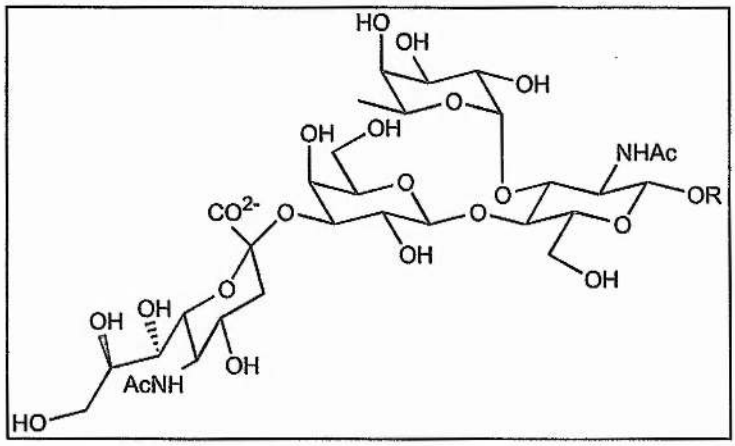


**Figure 1.8: The Sialyl Lewis x Antigen**

R = glycoprotein or glycolipid

### 1.3.2 Sialyl Lewis x and its Role in Inflammatory Response

Under normal physiological conditions leukocytes (white blood cells) flow rapidly through the mammalian blood stream. In response to tissue damage a tetrasaccharide, sialyl Lewis x ($sLe^x$) is expressed on the surface of leukocytes. This carbohydrate interacts with a protein (E-selectin) present on the surface of the endothelium. The effect of this interaction is to slow the movement of leukocytes through the blood vessels, eventually leading to a 'rolling' effect. Firm attachment of the leukocytes can eventually occur leading to extravasation of the leukocytes into the damaged tissue (**Figure 1.9**) [12], [13], [14]. This process is part of the host's self defense mechanism, without which it is more susceptible to, among other things, bacterial infections.

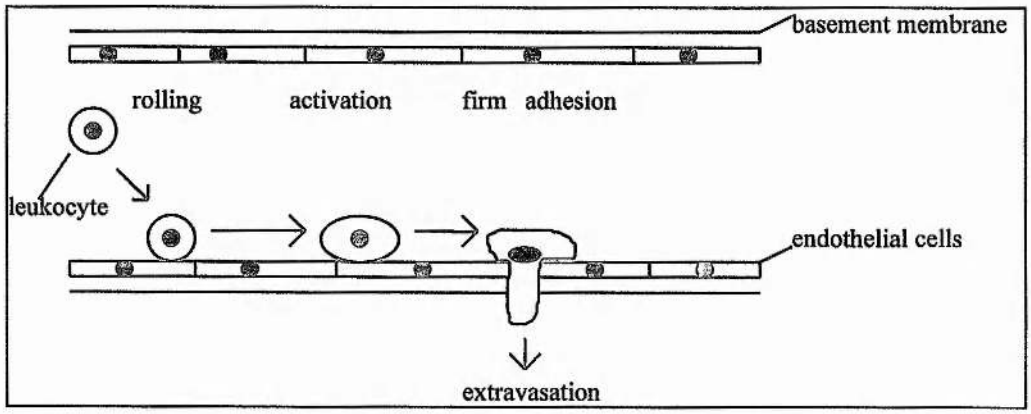


**Figure 1.9: Interaction of Leukocytes with Endothelial Cells.**

However, there are a series of acute and chronic disorders such as rheumatoid arthritis, psoriasis and acute and chronic inflammation, during which too many leukocytes are recruited to the site of injury resulting in severe damage to tissue. Therapeutic intervention by interruption of the interaction of the $sLe^x$ antigen with E-selectin might be possible.

### 1.3.3 Sialyl Lewis x and Cancer.

It has been found that tumor cells, in particular leukemia and carcinomas, have an increased concentration of *N*-acetyllactosamine residues terminating in $sLe^x$. The process of tumor formation is thought to be analogous to that of leukocyte recruitment during inflammatory responses. First the tumor cells flowing through the blood become attached to platelets. Similar to inflammation, this interaction of tumor cells with P-selectin is initiated

by the tetrasaccharide $sLe^x$. These tumor aggregates can be trapped in veins resulting in a release of cytokines such as interleukin-1 from the tumor cell resulting in extravasation of the tumor cell into the tissue [15]. Once the tumor cells grow in beneath endothelial tissue tumor metastasis is established [16].

### 1.3.4 Inhibition of $SLe^x$-Protein Interactions.

Sialyl Lewis x is obviously an important molecule in biological systems. Whilst being essential for the well being of the organism over-expression of this tetrasaccharide can have fatal consequences. Perhaps if the interaction of $sLe^x$ with carbohydrate - binding proteins could be interrupted, some of these unwanted physiological disorders could be prevented. One possible method for interrupting the carbohydrate-protein interactions described above involves synthesizing mimics of the $sLe^x$ antigen which would be recognized by the selectin, but which would prevent extravasation of the leukocyte into the damaged tissue. Work on this strategy is being carried out by a number of groups (see refs. [17] - [23]).

### 1.3.5 Biosynthesis of Sialyl Lewis x

An alternative strategy for altering the $sLe^x$ - E-selectin interaction during inflammatory response is to interrupt the biosynthetic pathway leading to $sLe^x$ formation. A method for achieving this is to inhibit the enzymes involved in the synthesis of $sLe^x$. Sialyl Lewis x is an α-2,3-linked sialylated tetrasaccharide which is structurally related to the $Le^x$ antigen (**Figure 1.8**). A cartoon of the biosynthetic pathway is shown in **Figure 1.10**. The first enzyme involved in the biosynthesis of $sLe^x$ is *N*-acetyglucosaminyltranserase which catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) to the terminus of an *N*-glycan. The second step is transfer of a galactose residue from uridine diphosphate galactose (UDP-Gal) to the 4-OH of the GlcNAc residue, catalyzed by UDP-Gal: β-1,4-galactosyltransferase (β-1,4-GalT). A sialic acid unit is then transferred from cytosine monophoshate sialic acid (CMP-sialic acid) to the 3 position of the galactose unit, catalyzed by CMP-sialic acid: α-2,3-sialyltransferase (α-2,3-SialylT). The final step in the biosynthesis is fucosylation. The enzyme catalyzing this step is α-1,3-fucosyltransferase

(α-1,3-FucT) and this results in transfer of a fucose residue from guanosine diphosphate fucose to the 3-OH of the GlcNAc moiety.

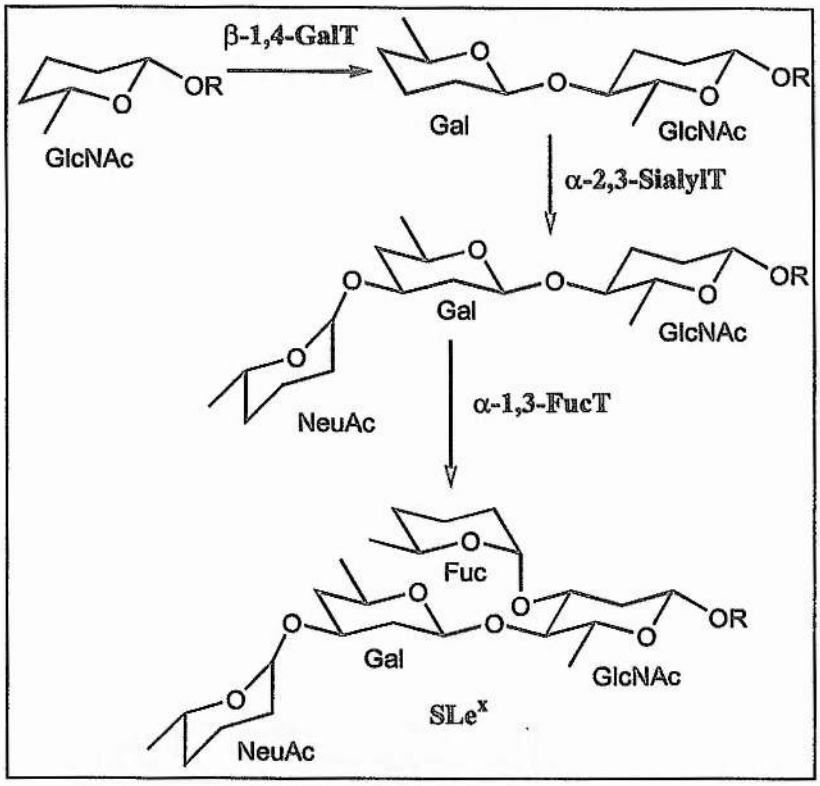


**Figure 1.10: The Biosynthetic Pathway Leading to sLe$^x$**

R = glycoprotein

There are four key enzymes participating in the biosynthesis of the sLe$^x$ antigen, thus there are four possible places to interrupt the biosynthetic pathway. Fucosylation is the final step in the biosynthetic pathway and would appear to be a good place to interrupt the biosynthesis with minimal effect on other biological processes.

## *1.4 Glycosyl Group Transfer*

The examples given in **Section 1.2** and **1.3** demonstrate the importance of oligosaccharides within living systems and as potential therapeutic targets for the treatment of cancer, infectious diseases and inflammation. The biosynthesis of oligosaccharides present on the surface of mammalian cells is controlled by the action of two classes of enzymes, the glycosyl hydrolases (glycosidases) and the glycosyltransferases. The processes catalyzed by these two classes of enzymes are essentially the same. Both are involved in the cleavage of glycosidic linkages between the anomeric carbon of a sugar and an oligosaccharide or a nucleoside diphosphate group. The liberated sugar can then be transferred to water (glycosidases) or to another nucleophilic acceptor (glycosyltransferases).

### 1.4.1 Non-Enzymatic Glycosyl Group Transfer

Nucleophilic substitution at the anomeric carbon of sugars is dominated by the participation of the lone-pair of electrons on the ring oxygen. This results in formation of a glycosyl cation (oxocarbonium ion). Nucleophilic attack on this cation results in formation of a new glycosidic linkage (**Figure 1.11**) (reviewed by Sinnot [24]). Oxocarbonium ions of this type are also thought to play a part in the enzyme catalyzed glycosyl transfer reactions.

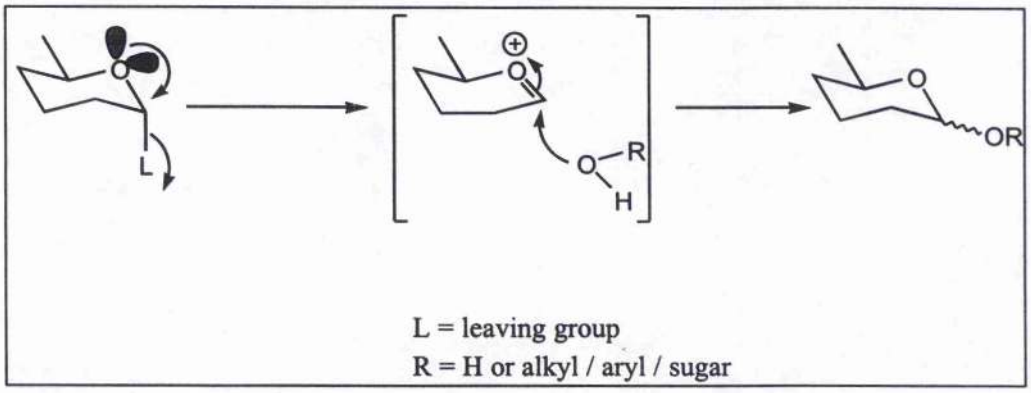


**Figure 1.11: Non-Enzymatic Glycosyl Group Transfer**

A review of methods for achieving chemical coupling of sugars can be found in **Chapter 3**.

### 1.4.2 Glycoside Hydrolases

There are several types of glycosidases which have been defined based on their modes of action. Enzymes which cleave glycosidic bonds at the non-reducing end of an oligosaccharide are known as exoglycosidases, whereas endoglycosidases cleave internal glycosidic linkages along a polysaccharide chain. A further classification is made based on the overall stereochemical outcome of the reaction catalyzed by glycosidases. Cleavage by a retaining glycosidase results in conservation of the anomeric configuration in the product and the mechanism is thought to involve a double inversion, as proposed by Koshland in 1953 (**Figure 1.12**) [24], [25].

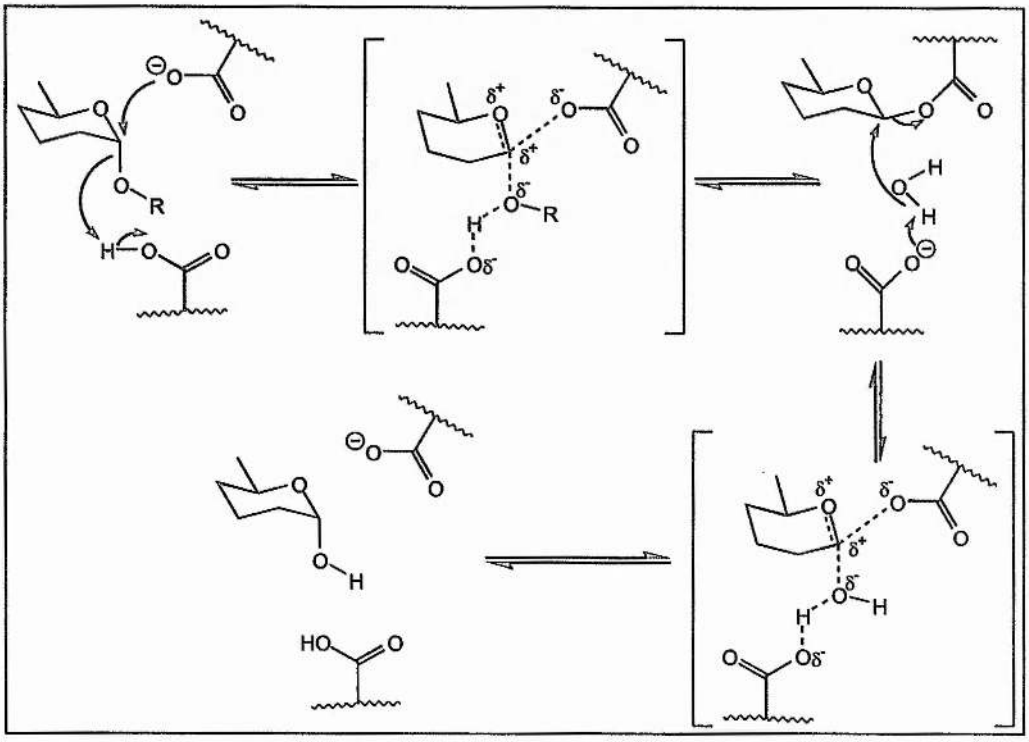

**Figure 1.12: Mechanism for Hydrolysis with Retention of Configuration**

Enzymes catalyzing hydrolysis yielding an overall inversion of configuration at the anomeric centre of the product are thought to proceed *via* a single inversion step and are known as inverting glycosidases (**Figure 1.13**).

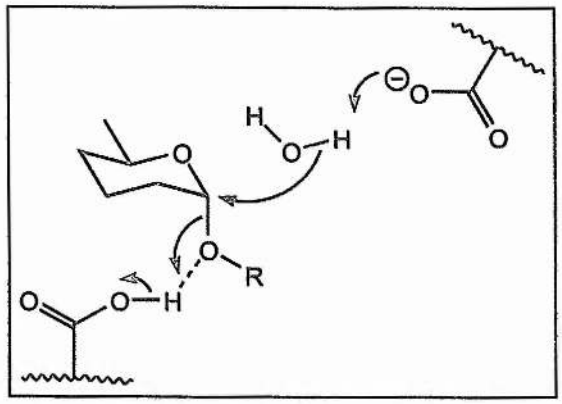

**Figure 1.13: Proposed Mechanism for Hydrolysis with Inversion of Configuration**

### *1.4.2.1 Glycosidase Inhibitors*

Specific inhibitors of glycosidases can yield information about the mechanism of the reaction and are also of therapeutic value, for example some act as antiviral agents by interrupting the biosynthesis of viral glycoproteins [26]. Many of the known inhibitors of glycosidases have structures which mimic the oxocarbenium ion transition state for glycoside hydrolysis. The first glycosidase inhibitors were monosaccharide derived δ-aldonolactones (e.g. D-gluconolactone) and glycosylamines (e.g. D-glucosamine) (**Figure 1.14**) which showed competitive inhibition of the glycosidases whose substrates they most closely resembled. More recently polyhydroxylated piperidines (e.g. 1-deoxynojirimycin), pyrrolidine (e.g. 2,5-bis(hydroxymethyl)-3,4-dihydroxypyrrolidine) and indolizine (e.g. castanospermine and swainsonine) derivatives (**Figure 1.14**) have been observed to inhibit glycosidases [27]. The aldonolactone derivatives are thought to act by mimicking the half chair conformation believed to be the intermediate in the mechanism of retaining glycosidases. The glycosylamines and aza sugars mimic the partial positive charge of the putative transition state of the enzyme.

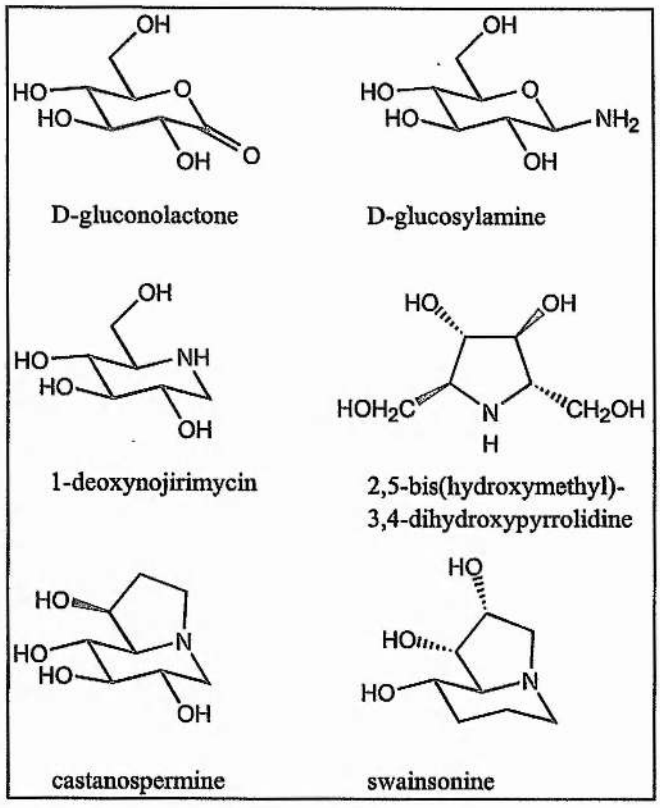


**Figure 1.14: Known Glycosidase Inhibitors**

A new class of glycosidase inhibitors are the amidines which are thought mimic the half chair conformation and the charge of the transition state intermediate as can be seen in **Figure 1.15** [27].

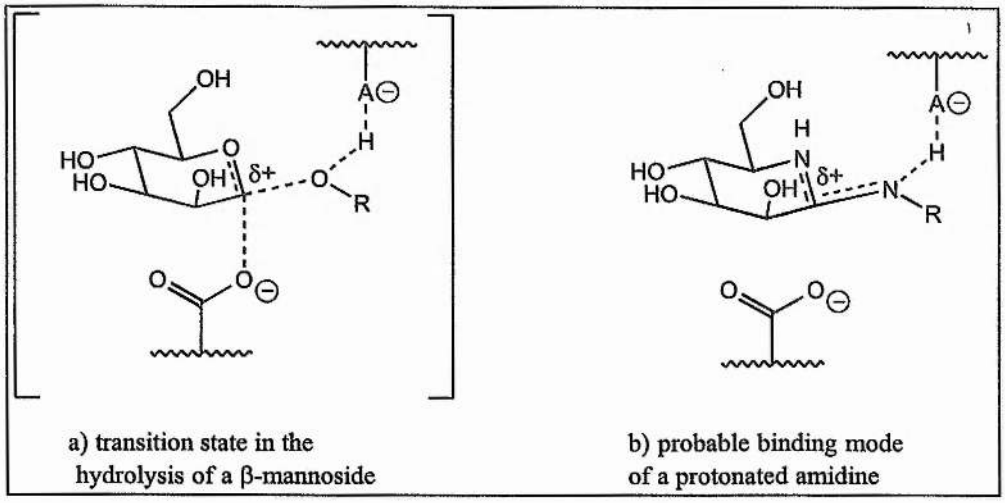


**Figure 1.15: Proposed Mode of Binding of a Protonated Amidine to a Mannosidase [28].**

### 1.4.3 Glycosyl Transferases

An alternative strategy for interrupting the biosynthesis of complex glycoconjugates in mammalian systems is to inhibit the enzymes involved in the formation of glycosidic linkages. This class of enzymes is known as glycosyltransferases. The reaction catalyzed by these enzymes is the transfer of a monosaccharide unit from a nucleotide donor to the hydroxyl group of an acceptor sugar (**Figure 1.16**).

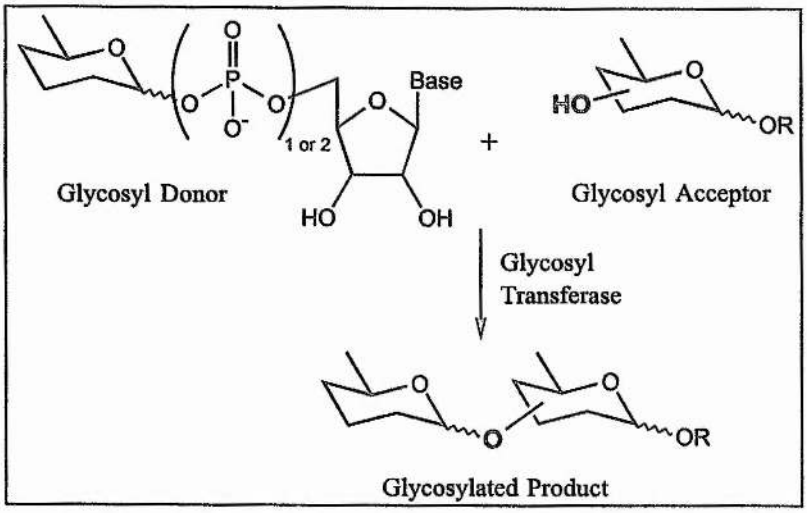


**Figure 1.16: Glycosyl Transferase-Catalyzed Glycoside Synthesis**

Glycosyltransferases are highly specific both in terms of the acceptor substrates used and in maintaining the stereo- and regio- control of the reaction. There are two distinct types of glycosyl transferase classified according to the stereochemical outcome of the reaction. The overall stereochemistry at the anomeric centre of the donor sugar is conserved during the reaction catalyzed by retaining enzymes. The second class of glycosyl transferase are inverting enzymes which effect an overall inversion of stereochemistry at the anomeric position of the donor. In order to account for all the different linkages and configurations of oligosaccharides found in mammalian systems there would need to be over one hundred different glycosyltransferases.

The transglycosidases are also capable of forming glycosidic linkages. Similar to the glycosidases described in **Section 1.4.2** these enzymes are responsible for the cleavage of glycosidic bonds between sugar residues, but like the glycosyltransferases, rather than transferring the sugar unit to a water nucleophile, a new glycosidic linkage is formed. For

example, the trypanosome *trans*-sialidase is capable of transferring a sialic acid residue from a pre-formed glycoconjugate to an oligosaccharide [29].

### *1.4.3.1 Mechanism of Glycosyl Transfer*

Understanding the exact mechanism for a glycosyl transferase is a non-trivial task due in part to the lack of crystal structures for any of the enzymes of this class. In an attempt to investigate the mechanism of glycosyl transfer Hindsgaul and co-workers [30] synthesized non-natural acceptor substrates for a variety of enzymes which had the hydroxyl group to be glycosylated replaced by a proton. The rationale behind this was that the transferase might still bind the deoxy substrate but that subsequent glycosylation would not then occur. The results of the study showed that for some glycosyl transferases the deoxy compounds were inhibitors of the enzyme (for example, β-1,6-GlcNAcT V). More interestingly however was the observation that for certain transferases (for example, β-1,4-GalT, α-1,3-FucT), no binding to the enzyme was observed when the deoxy compounds were used in the assay. This suggests that for some transferases there is a critical hydrogen bond between the enzyme active site and the hydroxyl group to be glycosylated on the acceptor molecule (**Figure 1.17**). The work carried out previously with glycosidases suggested that the active site of the enzyme may contain a general base such as the carboxylate side chain of glutamic or aspartic acid [30].

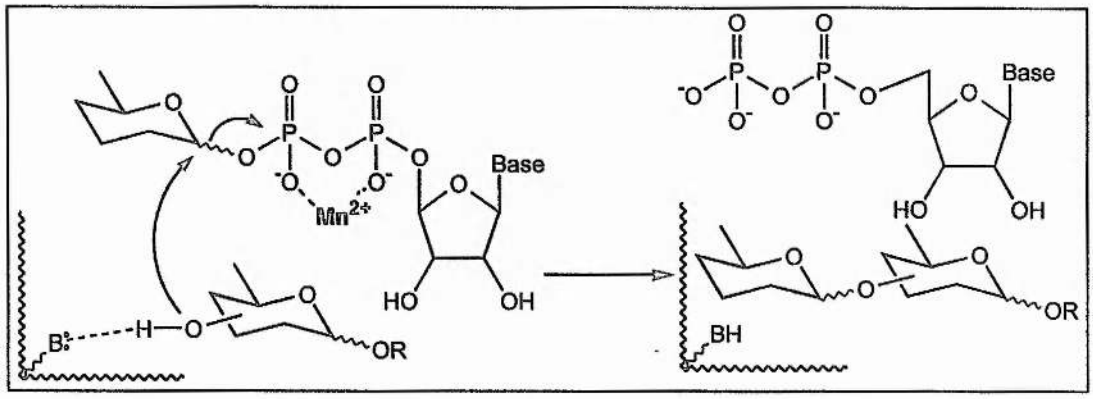


**Figure 1.17: Critical Hydrogen Bond Interaction**

-the key hydrogen bond may play a role...

If this model is correct potential acceptor analogue inhibitors could be designed which would have a positively charged moiety at the position to be glycosylated. A charge-charge interaction would ensue preventing glycosyl transfer from occurring. Indeed, much work has been carried out into design and synthesis of amino acceptor analogues as inhibitors of glycosyltransferases similar to the work carried out previously with glycosidases. The enzyme *N*-acetyllactosamine α-1,3'-galactosyltransferase (α-1,3-GalT) for example is strongly inhibited by the 3'-amino acceptor analogue (**Figure 1.18**) [31]. Another example is inhibition of the transferases α-1,3-GalT and α-1,3-GalNAcT, involved in the biosynthesis of the blood group antigens A and B, by their respective 3-amino acceptor analogues [32].

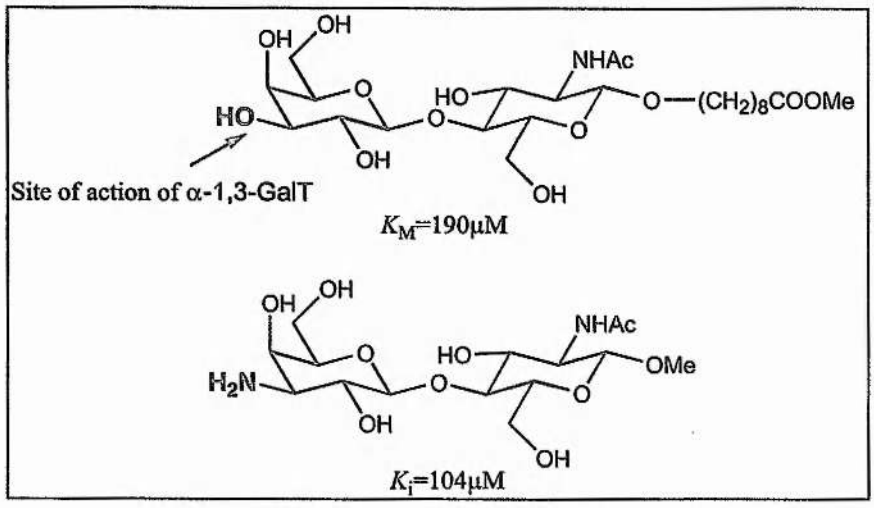


**Figure 1.18: A Potent Inhibitor of α-1,3-GalT**

However, there are enzymes that are not inhibited by their amino acceptor analogue. For example the amino acceptor analogue for bovine β-1,4-galactosyltransferase was a poor inhibitor for the enzyme and was in fact an apparent substrate (**Figure 1.19**) [33].

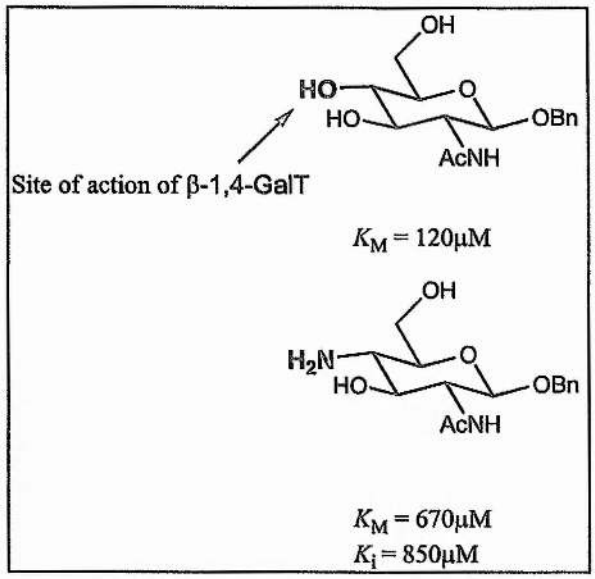


**Figure 1.19: Substrates for Bovine β-1,4-Galactosyltransferase**

### 1.4.4 Fucosyltransferase

Fucose residues are a common feature of cell surface carbohydrates. The enzymes responsible for catalyzing formation of fucosides are known as fucosyltransferases. There are seven distinct fucosyltransferases that are involved in forming different linkages with different acceptors. The most common fucose linkages found are α-1,2-, α-1,3- and α-1,4-. Each enzyme class differs in its tissue distribution, substrate specificity, cation sensitivity and its control of regiochemistry and anomeric configuration. However all fucosyltransferases are involved in catalyzing transfer of a fucose residue from guanosine diphosphate (GDP-fucose) to an acceptor molecule.

#### *1.4.4.1 α-1,2-Fucosyltransferase*

α-1,2-Fucosyltransferase (α-1,2-FucT) catalyzes the transfer of fucose from GDP-Fuc to the 2-OH of an acceptor substrate. An example is GDP-Fuc Gal-β-R α-1,2-FucT which is one of the enzymes involved in the biosynthesis of the blood group O antigen. The acceptor is the terminal galactose of a glycoprotein. The mechanism of transfer is not fully understood but it is thought that the inversion of anomeric configuration results from a direct $S_N2$ type displacement of GDP by the acceptor hydroxyl group (**Figure 1.20**) [34].

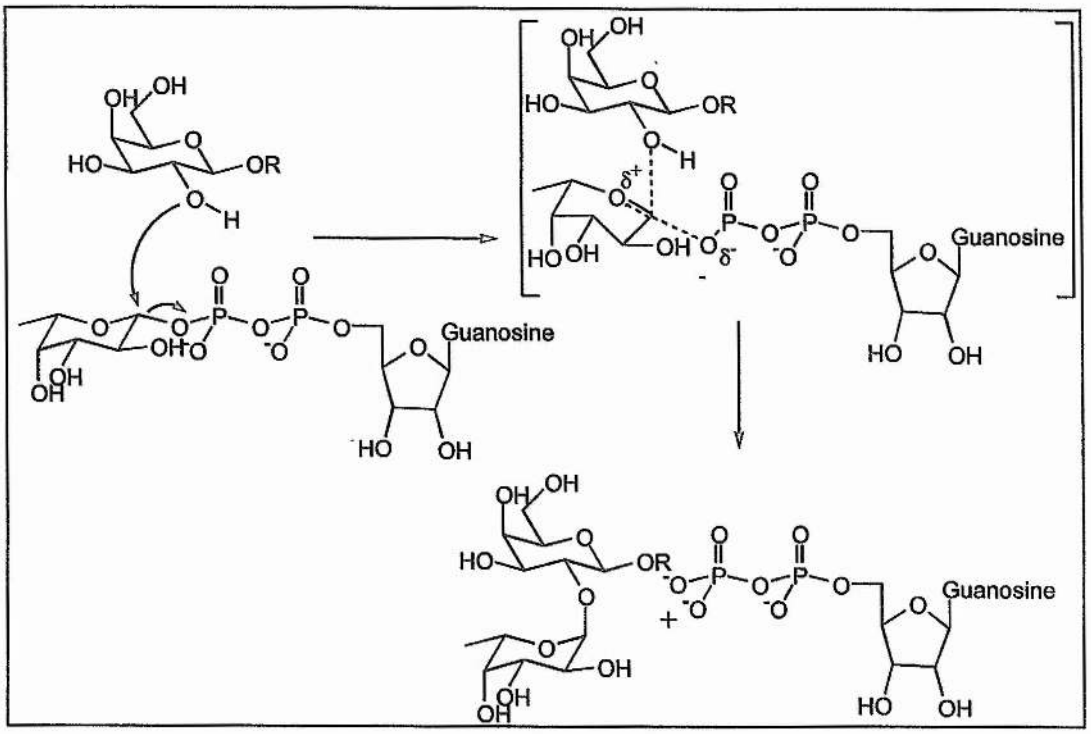


**Figure 1.20: Postulated Mechanism for α-1,2-FucT**

### *1.4.4.2 α-1,3-Fucosyltransferase*

Many carbohydrates on the surfaces of cells contain fucose residues in α-1,3-linkages. The formation of these linkages is catalyzed by α-1,3-fucosyltransferase enzymes which have been detected in a wide range of tissue types in mammalian systems [35] and have also been detected in plants, insects, bacteria (e.g. *Helicobacter pylori*) and snails (for review see [36]). The reaction catalyzed by α-1,3-FucT is the transfer of fucose from GDP-Fucose to the 3-OH of an acceptor sugar commonly 2-acetamido-2-deoxy-D-glucose (GlcNAc).

Five distinct types of human α-1,3-FucT have been cloned, III, IV, V, VI and VII, all of which have been shown to have different acceptor sugar specificities ( **Table** 1-2). One of these enzymes is unique as it can transfer a fucose residue to both type I chains (Gal-β-1,3-GlcNAc-β-OR) and the type II chains (Gal-β-1,4-GlcNAc-β-OR). It is therefore capable of catalyzing the formation of two different glycosidic linkages (α-1,3- or α-1,4-). This enzyme is known as the Lewis α-1,3/4-FucT III. It has the broadest acceptor

specificity range found among the human α-1,3-FucT's and can accept both sialylated and non sialylated substrates [37], [38].

| Enzyme | Tissue Distribution | Substrates | Products |
|---|---|---|---|
| α-1,3-FucT III | human milk, gall bladder, kidney, colon | type I, type II, sialyl type I + II, fucosyl type I + II, lactose, 2-fucosyl-lactose | $Le^a$, $sLe^a$, $Le^b$, $Le^x$, $sLe^x$, $Le^y$ |
| α-1,3-FucT IV | brain, myleoid cells | type II, sialyl type II | $Le^x$, $sLe^x$ |
| α-1,3-FucT V | plasma, human milk, liver | type II, sialyl type II | $Le^x$, $sLe^x$, $sLe^y$ |
| α-1,3-FucT VI | plasma, kidney, liver, colon | type II, sialyl type II, fucosyl type II | $Le^x$, $sLe^x$, $sLe^y$ |
| α-1,3-FucT VII | leukocytes | sialyl type II | $sLe^x$ |

**Table 1-2: Biochemical Properties of Human α-1,3-Fucosyltransferases.**

α-1,3-FucT IV - VII differ from III in that they cannot form α-1,4-linkages. α-1,3-FucT IV has been found to have the narrowest acceptor specificity range, having little or no activity with sialylated acceptors. It appears therefore to be ineffective in synthesizing sialyl Lewis x [3]. It is thought α-1,3-FucT V is responsible for the terminal step in the biosynthesis of Lewis x and sialyl Lewis x [39]. α-1,3-FucT V and VI have similar substrate specificities however α-1,3-FucT VI is more restricted and shows poor activity with Fuc-α-1,2-Gal-β-1,4-GlcNAc-R [3]. The final enzyme in this group, α-1,3-FucT VII, was the last to be cloned and is reported to give rise to cell surface $sLe^x$ expression but not $Le^x$ [40]. Recent evidence suggests that α-1,3-FucT VII participates in the biosynthesis of $sLe^x$ in leukocytes [41].

### *1.4.4.3 Mechanism of α-1,3-FucT V*

Wong and co-workers [42], [39], [43] have carried out extensive studies on the mechanism of human α-1,3-FucT V. Transfer of fucose has been demonstrated to occur with inversion of anomeric configuration. The mechanism which was put forward by Palcic and co-workers [44] for α-1,2-FucT postulated an ion pair intermediate (**Figure 1.20**). It is possible that α-1,3-FucT's work in a similar manner, the hydroxyl of the acceptor sugar displacing GDP from the donor. The active site base could assist in this by helping to deprotonate the hydroxyl of the acceptor. This mechanism is similar to that proposed for glycosidases [25]. Support for a process with this type of transition state comes from the observation that aza-sugars which mimic the charge of the transition state are inhibitors of α-1,3-FucT V and that in the presence of GDP they apparently become more potent inhibitors suggesting that a mimic of the transition state is forming in the assay [45]. Inhibition studies also demonstrated that GDP was a non-competitive inhibitor of α-1,3-FucT V and that at high concentrations of GDP-fucose potent substrate inhibition was observed suggesting that glycosyl transfer in α-1,3-FucT V involves a sequential mechanism with GDP-fucose binding first and the product GDP releasing last [42]. Wong and co-workers [39] also carried out pH-rate profile studies which supported the hypothesis for a catalytic active site base. The pKa of this base was found to be 4.1, comparable to that of a carboxylic acid. Solvent kinetic isotope studies have demonstrated that there is one transferable proton involved in the catalytic transition state. More recently inhibition studies have been carried out using an unnatural 2-fluoro-2-deoxy GDP-fucose derivative. The strategy for this comes from the work carried out previously with glycosidases [46]. This donor substrate was found to be a competitive inhibitor of the enzyme (Ki = 4.2 μM). This result differs from that obtained for glycosidases, the 2-fluoro derivatives were found to be slow substrates for retaining glycosidases. These observations led Wong and co-workers [43] to conclude that glycosidic cleavage occurs prior to nucleophilic attack for the enzyme α-1,3-FucT V.

## *1.5 Divalent Cations and Enzymatic Glycosyl Transfer*

### 1.5.1 The Role of Metal Ions in Enzymatic Catalysis

It is well established that metal ions play an important and diverse role in biological processes. For example metal ions are involved in such processes as oxidation and oxygen transport. An example is the zinc containing enzyme carbonic anhydrase which is responsible for the hydration of carbon dioxide to yield bicarbonate. It is thought that the nucleophile in the reaction is a zinc bound hydroxide moiety which attacks carbon dioxide forming a zinc bound bicarbonate ion (**Figure 1.21**) [47].

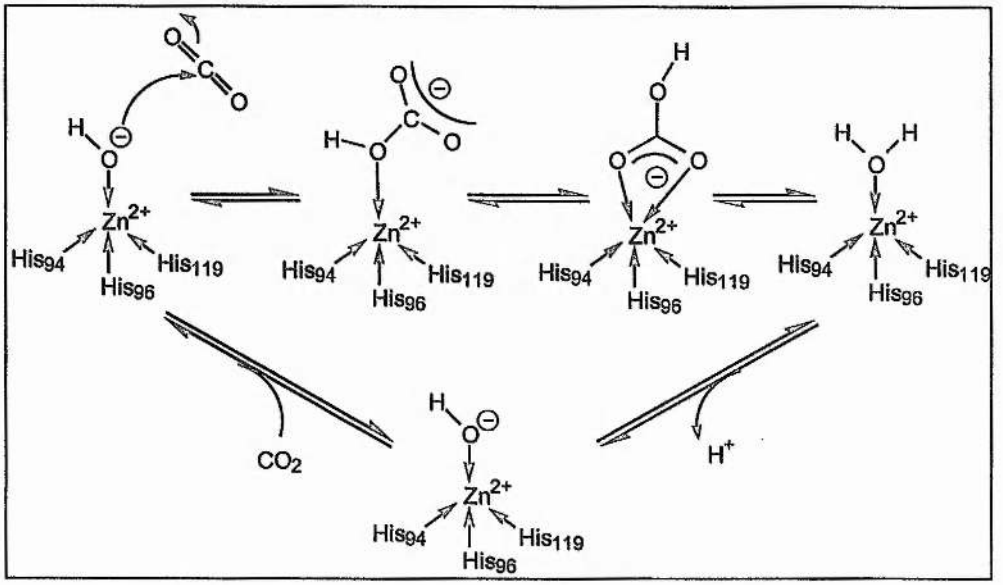


**Figure 1.21: Proposed Mechanism of Carbonic Anhydrase**

In this manner the zinc is acting by effecting a reduction of pKa of the bound water molecule, thereby increasing the nucleophilicity of the oxygen atom. A related mechanism has been proposed for inositol monophosphatase which utilizes a $Mg^{2+}$ bound water molecule as the nucleophile in the hydrolysis of inositol monophosphate. An added requirement for catalysis with this enzyme is the carboxylate side chain of glutamic acid [48], [49].

### 1.5.2 Role of Metal Ions in Glycosyl Transferases

It has been established that for some glycosyl transferases, for example, β-1,4-GalT, divalent metal ions play an important role in the mechanism. In many cases maximum *in vitro* activity is observed in the presence of divalent manganese cations [50]. During studies with β-1,4-GalT isolated from bovine milk, Powell and co-workers [51] identified that there were two distinct metal ion activation sites. Site I is occupied by a tightly bound $Mn^{2+}$ ion and can be satisfied with 10-20 μM concentrations, an attainable *in vivo* concentration range [50]. The $Mn^{2+}$ ions occupying Site II however are bound less tightly and saturation requires a concentration of 10-20 mM, a far greater concentration than is likely to be attained *in vivo* (*in vivo* $Mn^{2+}$ concentration is close to or less than $10^{-8}$ M almost everywhere inside or outside cells) [52], [53]. Studies have shown that for many glycosyl transferases the $Mn^{2+}$ ions can be replaced by other metal ions having an octahedral geometry such as $Mg^{2+}$, $Ca^{2+}$ and $Co^{2+}$. This substitution, however usually has an adverse effect on the rate of glycosyl transfer. This reduction in rate can be explained if it is assumed that these alternative metal ions occupy Site II and that there is incomplete occupancy of Site I by endogenous ions ($Mn^{2+}$). The greater rate observed when $Mn^{2+}$ ions are used could be due to their ability to satisfy both sites on the protein.

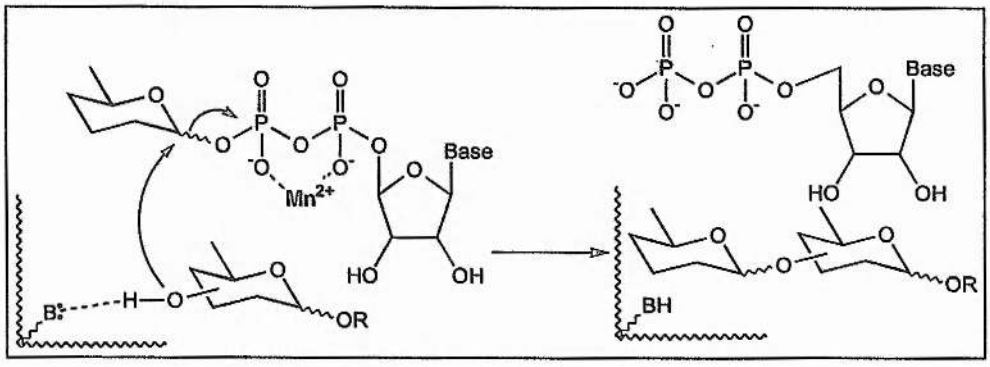


**Figure 1.22: Proposed Role of the 'Catalytic' $Mn^{2+}$ ion in Glycosyl Transferases.**

The $Mn^{2+}$ ion at Site I is thought to have a structural role due to the tight binding observed, whereas the role of the metal ion at Site II is assumed to be catalytic. It is believed that this 'catalytic' $Mn^{2+}$ ion coordinates the pyrophosphate group of the NDP-sugar substrate

(**Figure 1.22**). For β-1,4-GalT kinetic studies have suggested that an enzyme-$Mn^{2+}$-UDP-galactose complex may exist [51].

### *1.5.2.1 $Mn^{2+}$ Ions in α-1,3-Fucosyltransferase.*

Wong and co-workers [39] have shown that there is a manganese ion dependence for the enzyme α-1,3-FucT V. Kinetic studies have revealed that in order to attain half maximal activity it is necessary to have 6.1mM $Mn^{2+}$ ion concentration (GDP-Fuc 0.050mM, LacNAc 20mM). Similar to the studies described above, the endogenous metal ions were dialyzed from the protein and other metal ions were used in kinetic experiments. It was found that $Mn^{2+}$ ions could be replaced with $Ca^{2+}$, $Co^{2+}$ or $Mg^{2+}$, giving relative rates with respect to $Mn^{2+}$ of 63%, 56% and 53% respectively. From these studies it was concluded that for α-1,3-FucT V there is a preference for divalent metal ions that can adopt an octahedral geometry. The model postulated for this $Mn^{2+}$ ion dependence, was coordination of the metal ion to the pyrophosphate group of the donor substrate based on the fact that for both β-1,4-GalT and α-1,3-FucT the following order of inhibition was observed: NDP > NMP > N.

### *1.5.2.2 An Alternative Role for $Mn^{2+}$ in Glycosyl Transferase Reactions?*

Although it is known that in certain glycosyl transferases the rate of transfer can be enhanced by the presence of divalent metal ions ($Mn^{2+}$), it is not clear what the mechanism of this cationic activation is. The current thought is that the 'catalytic' $Mn^{2+}$ coordinates the pyrophosphate group of the donor sugar (**Figure 1.22**). However, there is little direct experimental evidence to substantiate this theory. If the $Mn^{2+}$ ions involved in the mechanism of glycosyl transfer are associated with the acceptor substrate a mechanism similar to that for carbonic anhydrase or inositol monophosphatase could be proposed for glycosyl transferase enzymes. This would involve coordination of the $Mn^{2+}$ to the active hydroxyl group of the acceptor (**Figure 1.23**). The overall effect of this coordination would be lowering of the pKa of the hydroxyl group and thus increasing the nucleophilicity of the oxygen to attack the donor substrate. It has been shown from the work of Hindsgaul and co-workers [30] that there may be an active site base involved during glycosyl transfer. This

may also coordinate the $Mn^{2+}$ ions helping to orientate the metal ion for coordination to the acceptor substrate (**Figure 1.23**).

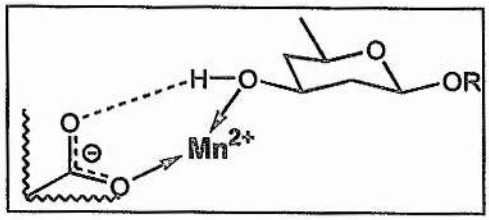


**Figure 1.23: Proposed Role of $Mn^{2+}$ in Glycosyl Transfer.**

Evidence to suggest that this type of mechanism could occur for glycosyltransferases comes from the proposed mechanism for oligosaccharyltransferase.

### *1.5.2.3 Oligosaccharyl Transferase*

One of the enzymes involved in the biosynthesis of *N*-linked glycoproteins is oligosaccharyltransferase. This enzyme facilitates the transfer of oligosaccharides onto the asparagine side chain of a protein (**Figure 1.24**, where X=O).

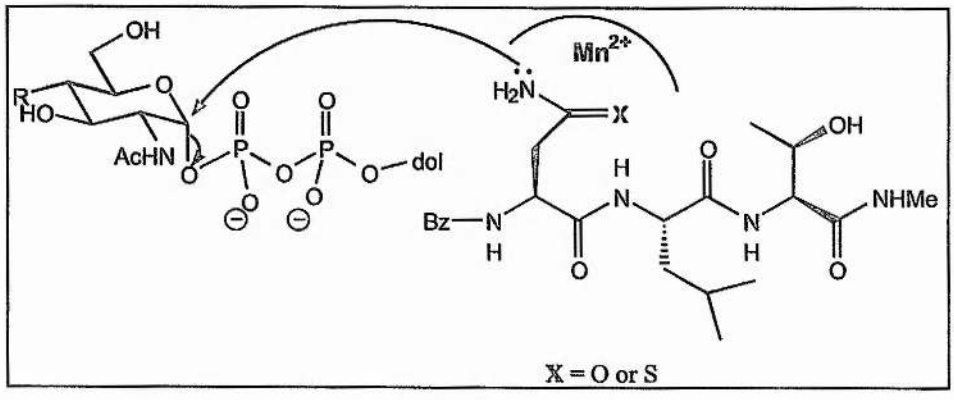


**Figure 1.24: Mechanism of Oligosaccharyl Transferase**

It is known that this enzyme has a dependence on divalent metal cations although the role of these cations is unclear. It was found that although $Mn^{2+}$ could be replaced by other divalent metal cations ($Ca^{2+}$, $Fe^{2+}$ and $Mg^{2+}$) this resulted in a 50% loss of activity. Imperiali and co-workers [54] argued that this preference for $Mn^{2+}$ ions suggests that the metal co-factor is not coordinated to the pyrophosphate group of the donor but is in close proximity to the acceptor substrate (**Figure 1.24**). If the metal ion coordinated the

pyrophosphate group it would be expected that replacement of $Mn^{2+}$ with $Mg^{2+}$ would result in an increase in activity due to the affinity of $Mg^{2+}$ for phosphate moieties. In an attempt to establish the role of the metal co-factor Imperiali and co-workers synthesized an unnatural substrate for the enzyme (**Figure 1.24**, where X=S). This substrate has the carbonyl group on the side chain of asparagine substituted by a thiocarbonyl. The rationale for this study was that the sulfur containing compound would have a different affinity towards the $Mn^{2+}$ cation. The thiol compound was found to have a similar $K_M$ (~ 380 μM) to that of the natural substrate but the $V_{max}$ was an order of magnitude lower. When alternative metal co-factors were used in the kinetic studies, it was found that the thiophilicity of the metal directly correlated with the observed rates of turnover, $Fe^{2+}$ ions giving the highest overall rate of transfer. It was also observed that when metals such as $Mg^{2+}$ (oxophilic) were used as co-factors poor rates of turnover resulted. Thus it was concluded that the metal co-factor was associated with the acceptor substrate and not the pyrophosphate group of the donor substrate.

### 1.5.3 Other $Mn^{2+}$ Containing Enzymes

#### *1.5.3.1 Fructose-1,6-Bisphosphatase*

Fructose-1,6-bisphosphatase (FBPase) is involved in the gluconeogenic pathway and catalyzes the conversion of D-fructose-1,6-bisphosphate to D-fructose-6-phosphate (**Figure 1.25**).

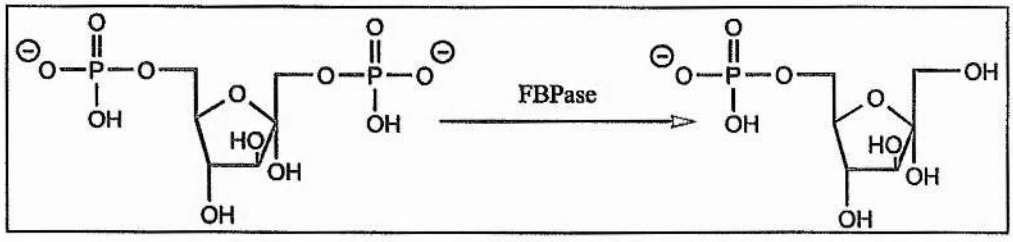


**Figure 1.25: Reaction Catalyzed by FBPase.**

The presence of divalent metal ions is essential for this reaction to occur. Divalent magnesium, manganese, zinc or cobalt can all be used effectively during catalysis. It is believed that there are between one and three metal ion sites. Only one $Mn^{2+}$ cation binds in the absence of substrate however two ions bind in the presence of substrate [49]. A crystal

structure of the enzyme bound to an inhibitor (2-OH replaced by H) shows the two $Mn^{2+}$ ions to be separated by 3.7 Å. The $Mn^{2+}$ ions are bridged by two carboxylate side chains of amino acids Glu-98 and Asp-118 (**Figure 1.26**).

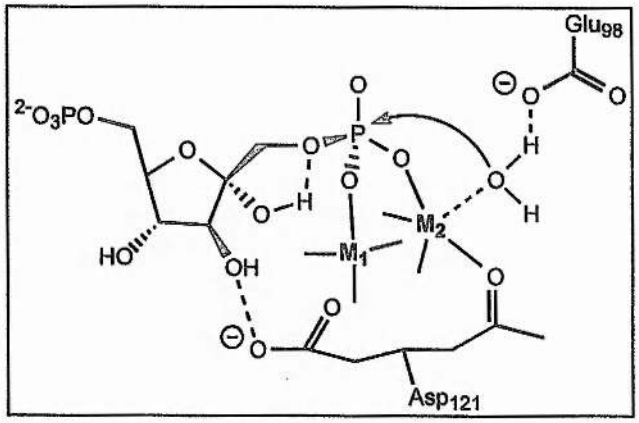


**Figure 1.26: Putative Mechanism of FBPase.**

The two metal ions ($M_1$ and $M_2$) are thought to orientate the substrate for attack by the water nucleophile and to stabilize the transition state. The pKa of the water molecule is reduced by coordination to $M_2$ facilitating in the general base deprotonation of the water molecule by Glu-97. Further support for this mechanism comes from the observation that substrates lacking 2-OH are resistant to enzymatic cleavage.

### *1.5.3.2 Arginase*

One of the enzymes involved in the disposal of nitrogenous waste from mammalian systems is arginase. This enzyme is responsible for the hydrolysis of arginine in the final step of the urea pathway (**Figure 1.27**).

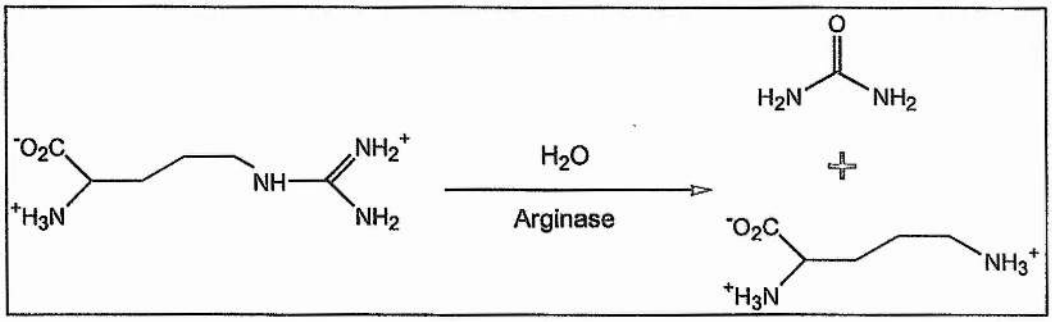


**Figure 1.27: Hydrolysis of Arginine**

Christianson and co-workers [55] recently published the crystal structure of arginase isolated from liver tissue. It was found that this enzyme contains a binuclear manganese cluster at the active site of the enzyme. Each subunit of the enzyme contains two spin coupled $Mn^{2+}$ ions that are an absolute requirement for activity. The separation of the metal centers was found to be 3.4-3.6Å however this distance narrows in the presence of inhibitors. This suggests that substrates are bound directly to the $Mn^{2+}$ site. This $Mn^{2+}$ - $Mn^{2+}$ distance is also consistent with the presence of one or more bridging carboxylate residues. Kinetics studies have revealed that there is a functional group with a pKa of 7.9-8.0 that is responsible for controlling the hydrolysis rate at the guanidinium carbon of the L-arginine substrate. This is consistent with an active site base which it is presumed deprotonates water forming the hydroxide nucleophile for the reaction (**Figure 1.28**). It is therefore assumed that arginase effects hydrolysis by lowering the pKa of a bound water molecule by forming a binuclear manganese cluster, thus increasing the nucleophilicity of the water molecule [56].

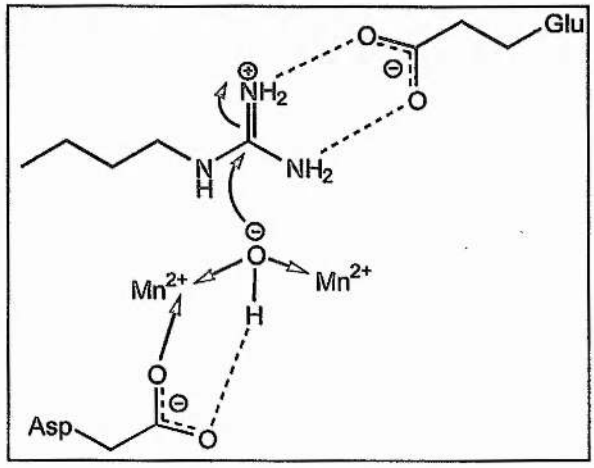


**Figure 1.28: Proposed Mechanism for Arginase**

## *1.6 Observations and Objectives*

It is evident from the examples shown that there are many similarities between enzymes containing divalent metal cation sites. For example many of the enzymes described have a requirement for an active site general base, commonly the carboxylate side chain of aspartic or glutamic acids. In some cases it has been shown that the metal ion plays an important role in the mechanism of the reaction and is not present purely in a structural capacity. Carbonic anhydrase for example utilizes the divalent metal ion ($Zn^{2+}$) to effect an overall lowering of the pKa of a bound water molecule. It has been established that divalent metal ions are important in glycosyltransferase reactions, in some cases it is an absolute requirement, however it is not clear what role these metal co-factors play. An additional similarity between glycosyltransferases and other $Mn^{2+}$ containing enzymes is the presence of an active site base which is thought to deprotonate the bound hydroxyl group. Perhaps it is possible for the $Mn^{2+}$ ions to coordinate the hydroxyl group of the acceptor substrate in a similar manner to that for arginase and FBPase. This would have an overall effect of increasing the nucleophilicty of the oxygen of the acceptor substrate towards attack on the nucleoside diphosphate donor.

Unpublished work by Palcic and co-workers (personal communication) has shown that perhaps the simple general base mechanism proposed by Hindsgaul and co-workers (**Figure 1.17**) does not tell the whole story for the mechanism of fucosyl transfer. A panel of compounds were synthesized whereby hydroxyl groups around the disaccharide acceptor substrate were systematically substituted with sulfate moieties [57]. The results obtained are shown in **Figure 1.29**. An unusual result was obtained for the 3-*O*-sulfate derivative. It was expected that this analogue would be a non-substrate due to the presence of a negative charge at the site of glycosylation. This would interfere with the general base activation of the acceptor molecule. However it was found that this unnatural analogue was in fact a substrate for the enzyme with kinetic parameters similar to those for the unsubstituted compound. Although this result does not prove or disprove the previously proposed mechanism for glycosyltransferases, it does suggest that perhaps the mechanism is more complex than previously supposed. The mechanism postulated in this thesis however can explain the results obtained for the 3-*O*-sulfate derivative.

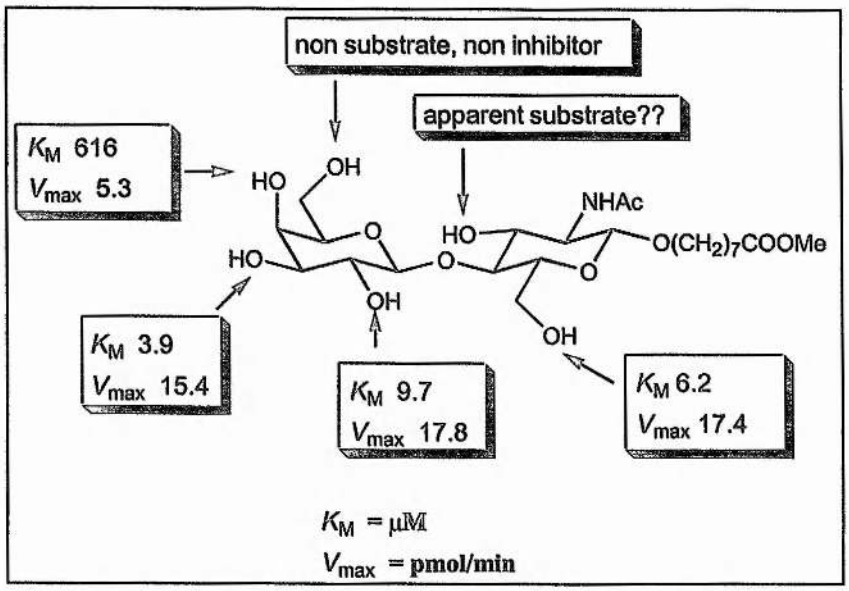


**Figure 1.29: Kinetic Data for Sulfated Acceptor Substrates with α-1,3-Fucosyltransferase (milk)**

The objective of this thesis was therefore to further investigate what role divalent metal ions have in the mechanism of α-1,3-fucosyltransferase. The strategy for achieving this objective was to synthesize unnatural acceptor analogues containing anionic functionalities at the site of glycosylation. These anionic groups should have a different affinity for the metal ion and thus by measuring the kinetic parameters of these substrates with the enzyme it may be possible to determine whether the $Mn^{2+}$ cation is associated with the acceptor substrates as postulated in this thesis.

The following chapter will discuss in more depth the strategy of work for this thesis. Chapter 3 will discuss the chemical synthesis of the target molecules undertaken during this project. Chapter 4 will contain the experimental details for the chemical synthesis. The results of the biological testing of these molecules will be presented in Chapter 5. Chapter 6 will draw some conclusions from the work carried out.

## *1.7 References*


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# *Chapter 2: Strategy of Work*

## *2.1 Design of Acceptor Substrates for α-1,3-Fucosyltransferases*

To design effective substrates for an enzyme it is necessary to consider the minimum substrate tolerated by the enzyme, the acceptor specificity and the sites important for enzyme recognition. It is known that α-1,3-FucT's are involved in the terminal step in the synthesis of $sLe^x$ terminating units of glycoconjugates and it has been shown that for most α-1,3-FucT's the minimum substrate tolerated by the enzyme is a disaccharide unit based on the Gal-β-1,4-GlcNAc core unit. The exception to this is α-1,3-FucT VII for which it is reported the trisaccharide NeuAc-α-2,3-Gal-β-1,4-GlcNAc is the minimum substrate tolerated [1]. Another exception is α-1,3-FucT III which accepts Gal-β-1,4-GlcNAc and Gal-β-1,3-GlcNAc disaccharides. Work carried out by Palcic and co-workers [2] has determined the sites important for enzyme recognition of an acceptor substrate for the Lewis α-1,3/4-FucT (human milk). These sites are shown in **Figure 2.1**.

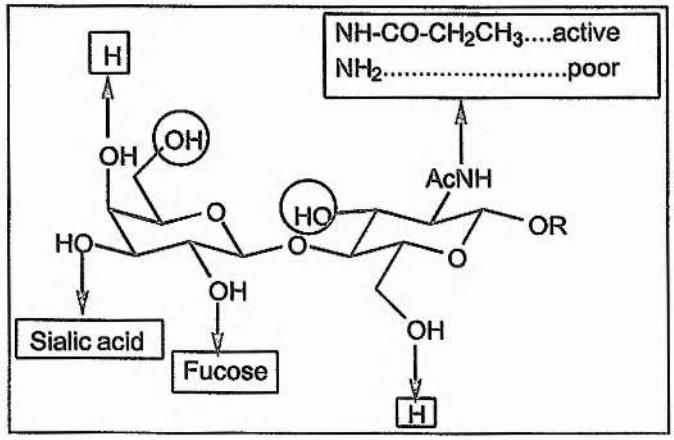


**Figure 2.1: The Sites Important for Recognition with Lewis α-1,3/4-FucT**

The sites circled are important for recogntion by the enzyme and modifications are not tolerated. The sites in boxes can be modified to some extent without inactivating the enzyme.

## 2.2 *Target Molecules*

The target molecules chosen to investigate the role of the divalent metal cation in the mechanism of fucosyltransferase catalyzed reactions are shown in **Figure 2.2.**

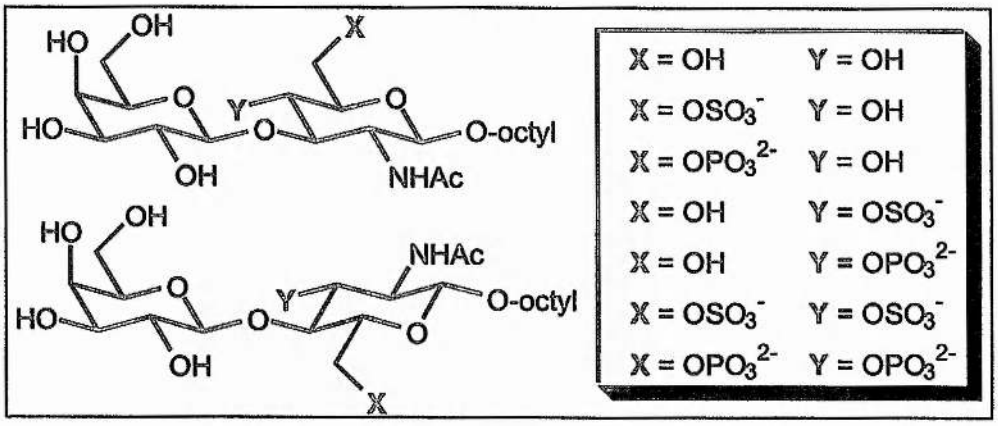


**Figure 2.2: Target Molecules**

These target molecules are disaccharides containing a D-galactose β-linked to either the 3-OH or 4-OH of an *N*-acetylglucosamine residue, thus forming type I and type II chains. All molecules contain a β-linkage to an octyl function, enabling purification of these molecules from biological mixtures by reverse phase chromatography using $C_{18}$ SepPak cartridges [3]. In the natural substrate the site occupied by this octyl chain would be a glycoprotein or glycolipid. It has been shown however that the biological activity of compounds containing a hydrophobic tail is not impaired and in fact for bovine β-1,4-GalT binding to the enyme has been demonstrated to be in the same range as for the natural glycans ($K_M$ ~ 150-200 μM) [4].

To investigate the role of the divalent metal cation in fucosyltransferase reactions it was decided to prepare sulfated and phosphorylated derivatives of octyl lacNAc. These anionic functionalities should have a different affinity for the metal ion than the corresponding hydroxyl group. From the observations made by Palcic and co-workers (unpublished) (**Section 1.6**), it was concluded that the sulfate group at the site of glycosylation was being fucosylated thus forming an unstable sulfate diester linkage. The rationale, then for preparing phosphorylated derivatives was to try and identify the product of the reaction. Glycosyl phosphate diester linkages are known in nature [5] and

should therefore be stable enough to allow isolation and characterisation of any phosphate diester product formed during the reaction.

It has been reported that the enzyme α-1,3-FucT VII requires a sialic acid at the 3'-OH for activity [1], therefore it was also decided to prepare the α-2,3-linked sialic acid derivatives of the compounds shown in **Figure 2.2**. Work currently ongoing in our laboratories uses a *trans*-sialidase enzyme from *Trypanosoma cruzi* to synthesize α-2,3-sialic acid glycosides and so these sialylated compounds were to be prepared using an enzymatic method (**Chapter 5**).

## *2.3 References*


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# *Chapter 3: Results and Discussion (Chemistry)*

## *3.1 Strategies in Carbohydrate Chemistry*

It is now well established that carbohydrates linked to proteins or lipids are involved in many important biological processes (Chapter 1). This increased appreciation of the role of these molecules in the biological sciences has resulted in a revival in carbohydrate chemistry. Synthetic analogues of biologically active oligosaccharides may provide essential information about structure-function relationships and may provide information about binding and interaction with other biopolymers. However synthesis of oligosaccharides is a non-trivial task due in part to the multi-functionality of such molecules. All monosaccharides involved in the synthesis of biologically active oligosaccharides contain at least three hydroxyl groups and the anomeric carbon can have two different configurations ($\alpha$ or $\beta$). Each coupling reaction has to be performed regio- and stereo-selectively and therefore the complexity of synthesis grows with the length of oligosaccharide chain.

### 3.1.1 Orthogonal Protection Strategies in Carbohydrate Chemistry

The presence of three or more hydroxyl groups in each monosaccharide residue forces the need for orthogonal protection strategies in oligosaccharide synthesis. During a coupling reaction it is often desirable to have only one free hydroxyl group present. In order to achieve this level of protection, many protection and deprotection steps might need to be employed. Careful choice of protecting group is therefore required when designing syntheses of oligosaccharides. For further information see [1].

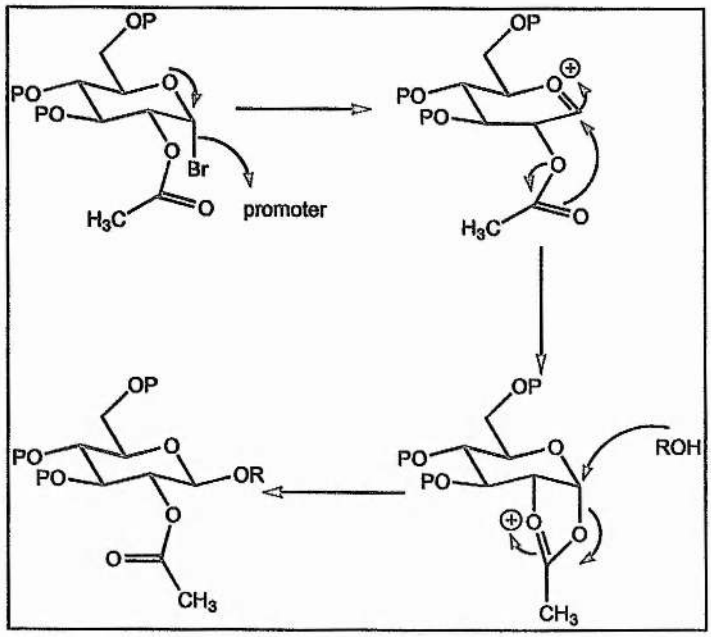
### 3.1.2 Glycosylation Methods

Only a brief outline of some of the strategies employed during glycoside synthesis will be presented here. For review information see [2].

#### *3.1.2.1 Neighbouring Group Participation*

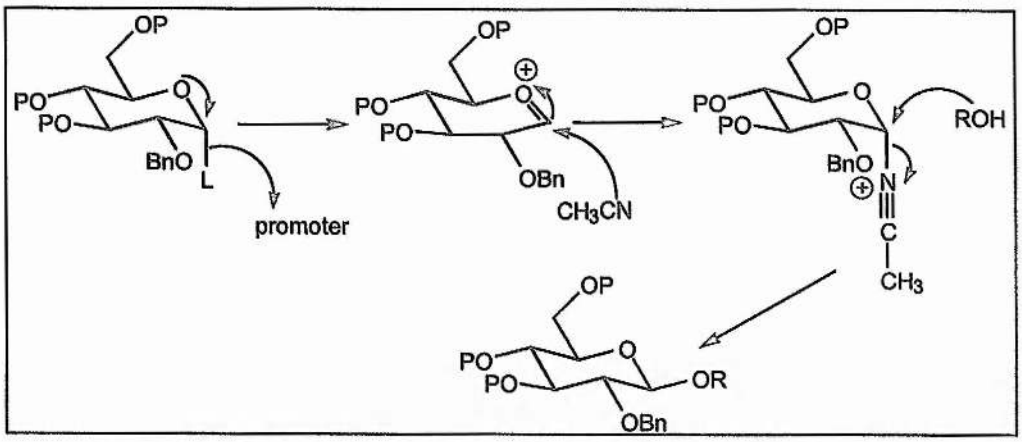
Coupling reactions in carbohydrate chemistry are usually achieved by reaction of the anomeric carbon of a fully protected sugar residue (glycosyl donor) with a free hydroxyl group of suitably protected sugar (glycosyl acceptor). Controlling the stereochemical outcome of the reaction is a difficult task and often mixtures of anomers are formed which

have to be separated by chromatography. To a certain extent the stereochemical outcome of a glycosylation reaction can be controlled by careful choice of the protecting group at position 2 of the glycosyl donor and the anomeric leaving group. The solvent and promoter used in the reaction are also important. For example, if a halide glycosyl donor is chosen which has an ester functionality at position 2, then a β-glycoside would be expected as the glycosylation product due to neighbouring group participation (**Scheme 3-1**). The incoming alcohol attacks in an $S_N2$ reaction from the top face of the molecule resulting in formation of the β-anomer exclusively.




**Scheme 3-1: Use of Participating Groups in Glycosylation Reactions**

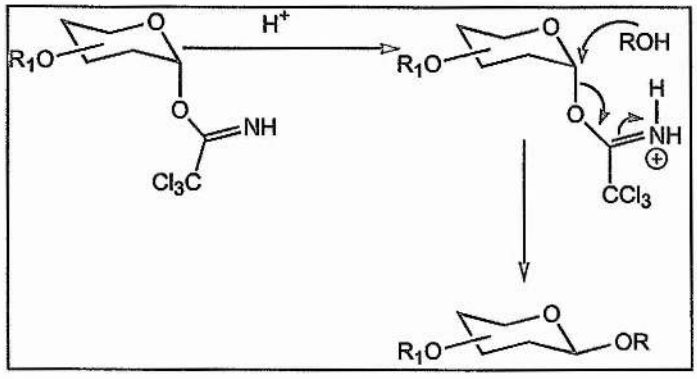
However if no participating group is present at position 2 the likely configuration of a glycosylation reaction would be α due to the anomeric effect [1]. This can be altered if a suitable solvent is used in the reaction, for example, acetonitrile which is able to participate in the reaction (**Scheme 3-2**).



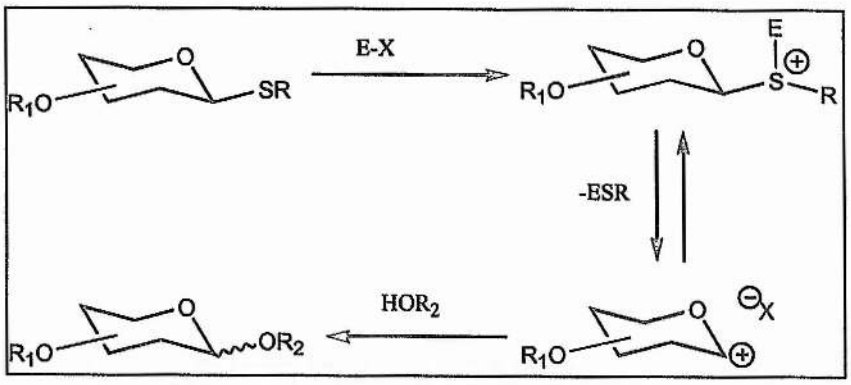

**Scheme 3-2: Acetonitrile as a Participating Solvent in Glycosylation**

### *3.1.2.2 Leaving Groups and Promoters*

Halides, usually chlorides or bromides, have traditionally been used as leaving groups in glycoside synthesis. With a careful choice of promoters, reaction conditions and type of protecting groups α- and β-anomers can be prepared with high selectivity. However, glycosyl halide donors are rather unstable and as such have proved unsuitable in convergent synthesis where blocks of oligosaccharides are coupled together. More recently trichloroacetimidates (**Scheme 3-3**) [3] and thioglycosides [2] (**Scheme 3-4**) have come into common use as glycosyl donors.




**Scheme 3-3: Glycosylation Reaction using Trichloroacetimidate Donor**




**Scheme 3-4: Activation of Thioglycosides with a Thiophilic Reagent (E-X) [2]**

The choice of promoter for a glycosylation reaction is dependent on the choice of glycosyl donor and must also take into consideration the reactivity of the glycosyl acceptor. **Table 3-1** shows some glycosyl donors and their respective promoters.

| Glycosyl Donor | Promoter |
|---|---|
| Br | $Br^-$, $HgCN_2$, $HgBr_2$, AgOTf |
| Cl | $HgCN_2$, $HgBr_2$, AgOTf |
| F | $SnCl_2$-$AgClO_4$ , AgOTf-$SnCl_2$ |
| SR | TfOH-NIS |
| OAc | $BF_3$, $SnCl_4$, TMSOTf |
| $(CH_2)_3CH=CH_2$ | TfOH-NIS, AgOTf-NIS, TESOTf-NIS |
| $HN=CCCl_3$ | $BF_3$-$Et_2O$, TfOH, TMS-OTf |
| SO.Ph | $Tf_2O$ |
| SCN | TMSOTf |
| SCS.(OEt) | $Cu(OTf)_2$ |

**Table 3-1: Commonly Used Glycosyl Donors and their Promoters**

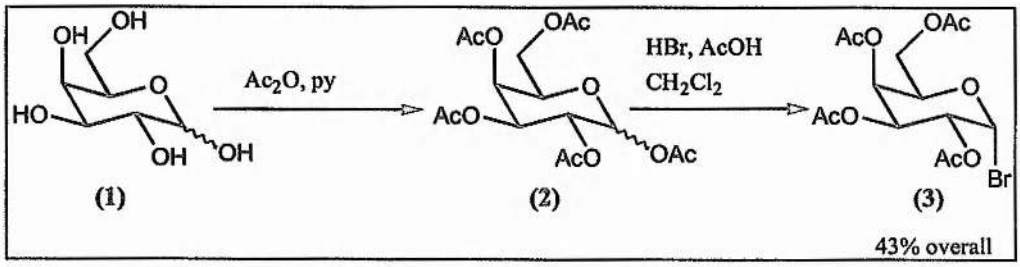
An alternative method for synthesizing oligosaccharides is to use an enzymatic method or a combination of chemical and enzymatic synthesis. This topic will be covered in **Chapter 4**.

## *3.2 Synthesis of Sulfate and Phosphate Mono- and Di-saccharides*

The target molecules shown in Chapter 2 were prepared from suitably protected *N*-acetyllactosamine derivatives. The monosaccharide starting materials used were *N*-acetylglucosamine and D-galactose.

### 3.2.1 Synthesis of the Glycosyl Donor:- 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl Bromide (3).

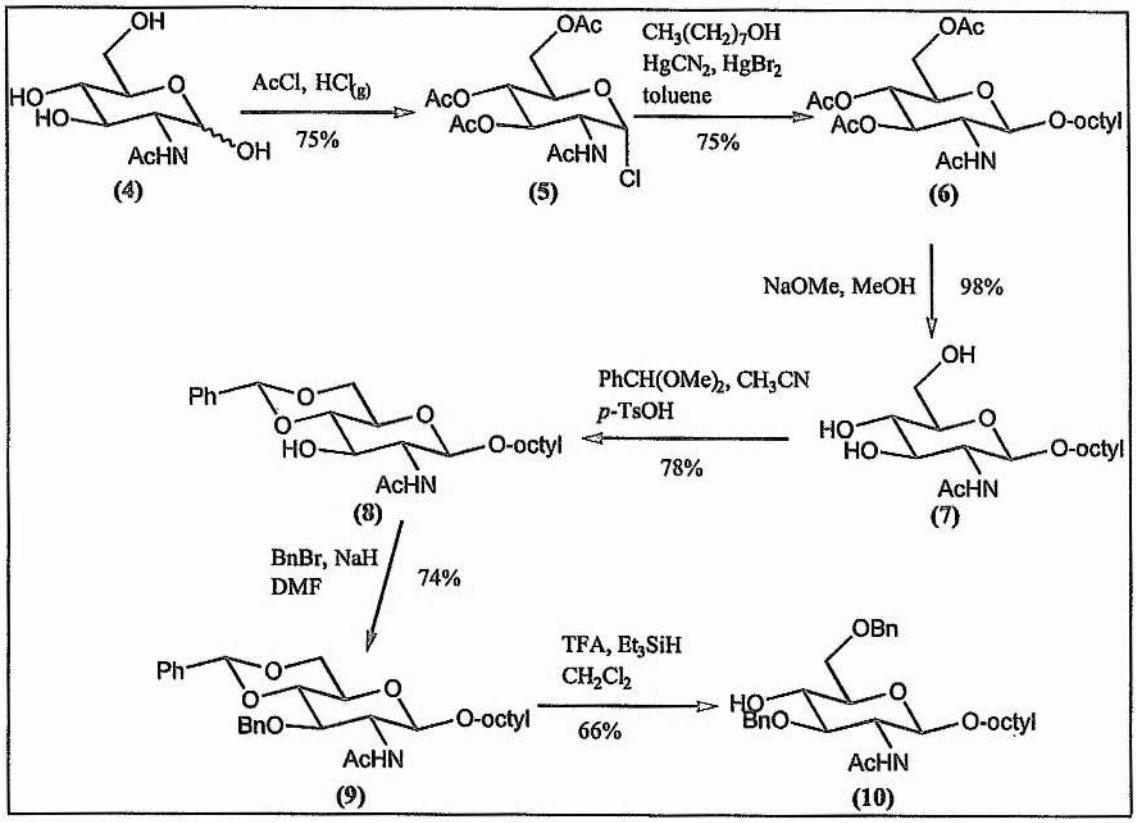
Penta-*O*-acetyl-α/β-D-galactose (2) was prepared by reaction of D-galactose (1) with acetic anhydride in pyridine (Scheme 3-5). The crude product of the reaction was converted to the glycosyl bromide (3) using hydrogen bromide in acetic acid.




**Scheme 3-5: The Synthesis of Acetobromogalactose (3)**

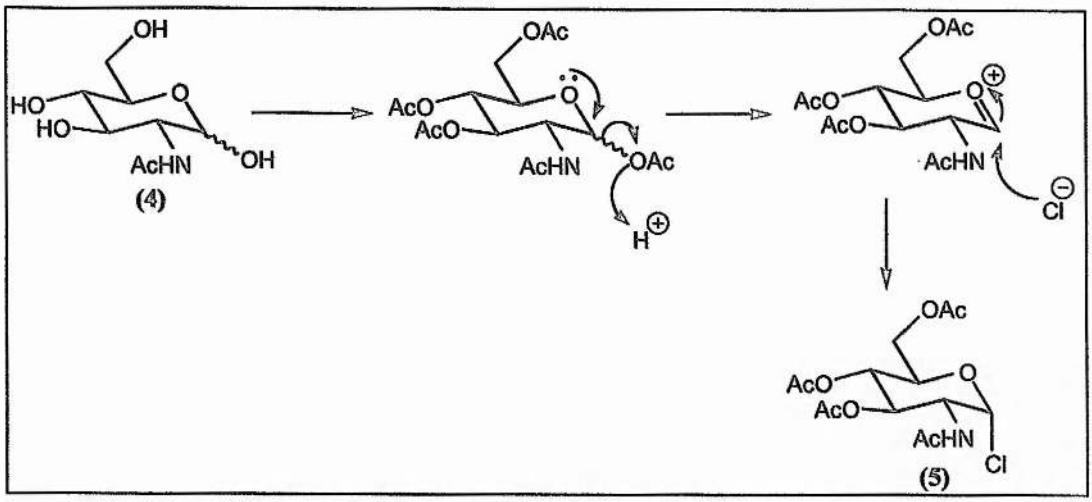
### 3.2.2 Synthesis of Glycosyl Acceptor for Chemical Coupling: Octyl 2-Acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (10)

The overall synthetic scheme for synthesis of octyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (10) is shown in Scheme 3-6.




**Scheme 3-6: The Synthesis of Octyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-Glucopyranoside (10)**

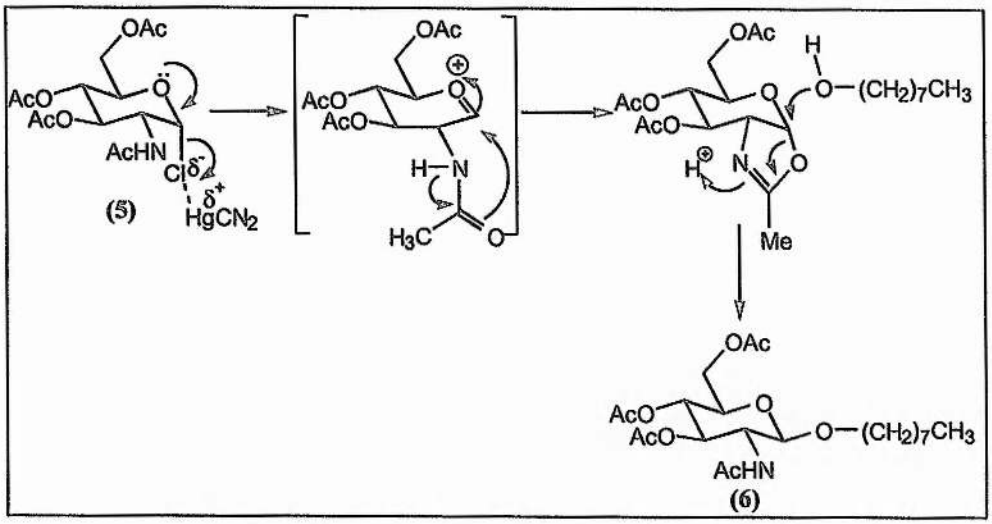
2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (5) was prepared by reaction of acetyl chloride with *N*-acetylglucosamine as reported by Horton [4]. However, the reaction was found to be slower than reported and typically gave lower yields (approximately 40%). Pentaacetylglucosamine is formed as an intermediate in the reaction releasing hydrogen chloride gas which attacks the anomeric carbon displacing acetic acid to form the *N*-acetylglucosaminyl chloride (5) (Scheme 3-7).




**Scheme 3-7: The Synthesis of 2-acetanido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl Chloride (5)**

The low yields and slow rate of the reaction were thought to be due to loss of the hydrogen chloride gas produced *in situ*. Several different procedures were attempted to try and generate more hydrogen chloride gas during the reaction and thus force the reaction to completion. Addition of acetyl chloride presaturated with hydrogen chloride gas to a flask containing *N*-acetyl glucosamine was found to be the best method of synthesizing the glycosyl chloride. The yields and reaction time were much improved compared with the initial attempts described.

Using the glycosyl chloride obtained above octyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (6) was prepared by a standard Koenigs-Knorr coupling procedure using mercury cyanide and mercury bromide as promoters [5]. The stereochemistry at the anomeric position was confirmed to be β by n.m.r. spectroscopy; the α anomer was not detected. The anomeric selectivity of this coupling reaction of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (5) to octanol can be predicted by considering the mechanism of the reaction. The mercury salts are halophilic and serve as promoters in the reaction by abstracting the chlorine at position 1 of the glycosyl chloride (5) (**Scheme 3-8**). The intermediate carbonium ion formed is stabilized by participation of the acetamido group at position 2. This leaves only the β face of the sugar residue open to an attack by octanol.




**Scheme 3-8: The Mechanism of the Coupling Reaction**

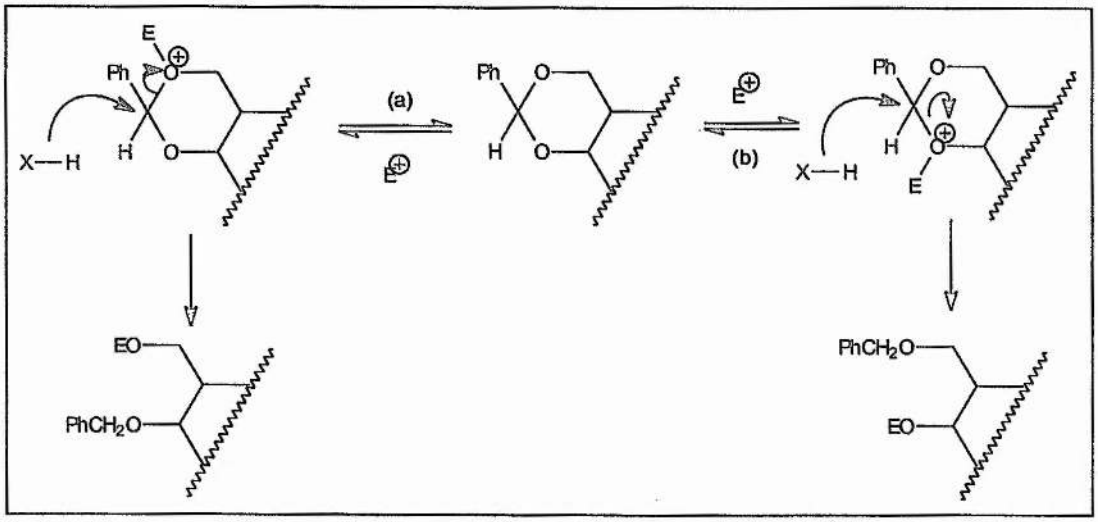
A problem encountered during the synthesis of octyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (6) was identifying the product by t.l.c.. The acetyl protected octyl *N*-acetyllactosamide had a similar $R_f$ value to the glycosyl chloride starting material. To confirm this observation a small amount of (8-methoxycarbonyl)octyl 2-acetamido-2-deoxy-β-D-glucopyranoside [5] was acetylated and the $R_f$ values of this and (5) were compared. This t.l.c. comparison confirmed that 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (5) and octyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (6) have similar $R_f$ values.

Compound (6) was deprotected using sodium methoxide and methanol to produce octyl 2-acetamido-2-deoxy-β-D-glucopyranoside (7) in quantitative yield (**Scheme 3-6**). Octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (8) was prepared by reaction of alcohol (7) with benzaldehyde dimethyl acetal and *p*-toluene sulfonic acid in acetonitrile. Alcohol (7) was insoluble in acetonitrile but dissolved on addition of benzaldhyde dimethyl acetal. A white precipitate of alcohol (8) instantaneously formed in the reaction vessel.

The 3-hydroxyl group of octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (8) was protected by reaction with benzyl bromide and sodium hydride in dimethylformamide to yield octyl 2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (9) . It was necessary to add only small aliquots of sodium hydride at

any one time and monitor the reaction carefully by t.l.c.. If the reaction is allowed to progress beyond formation of the monobenzyl derivative, the nitrogen of the acetamido group can become benzylated. This reaction was quenched by addition of water which resulted in compound (**9**) precipitating as a white solid [6].

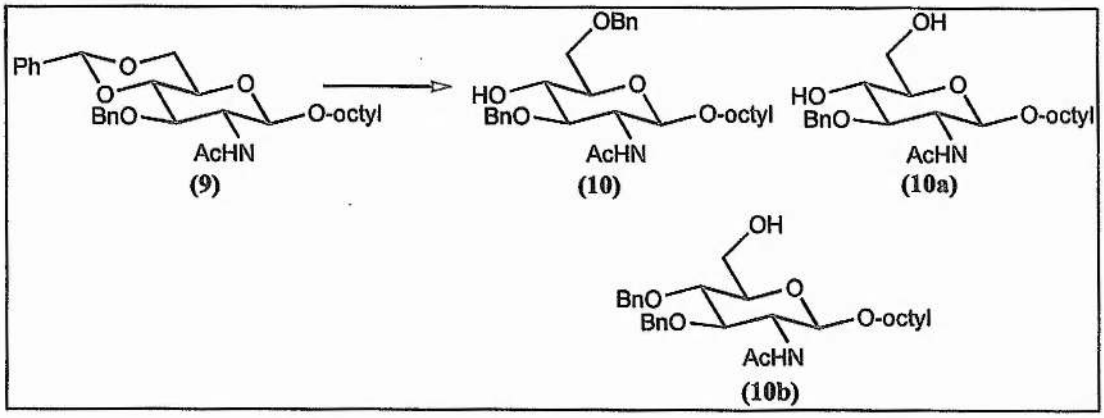
Using a selective acetal ring opening procedure it is possible to cleave benzylidene acetal protecting groups leaving the 6-position protected with a benzyl group while the 4-position is deprotected. The regioselectivity of such an acetal ring opening procedure can be explained by considering the mechanism proposed by Garegg and co-workers [7] as shown in **Scheme 3-9**. This mechanism shows two possible products from a reductive benzylidene acetal ring opening. Which alcohol (4- or 6-OH) is obtained depends on the nature of the electrophile, E. If E is, for example, a Lewis acid such as aluminium chloride, the reductive ring opening is likely to be directed towards path (a) due to the steric demand of the bulky electrophile. For the case where E is a proton, for example, in sodium cyanoborohydride reductions using HCl, the steric demand of the proton is less and so the direction of the equilibrium will depend on the relative basicities of the *O*-4 and *O*-6 and the reductive acetal ring opening is directed towards path (b).




**Scheme 3-9: Mechanism for Reductive Ring Opening of Benzylidene Acetals [7]**

Considering the mechanism for the regioselective benzylidene acetal ring opening reaction described above, the method required for the ring opening of compound (**9**) employs sodium cyanoborohydride and hydrogen chloride in tetrahydrofuran under

rigorously anhydrous conditions [7]. Octyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (**10**) was synthesized in only moderate yields (typically 60%) due to the competing formation of octyl 2-acetamido-3-*O*-benzyl-2-deoxy-β-D-glucopyranoside (**10a**), (**Scheme 3-10**).




**Scheme 3-10: The Acetal Ring Opening Reaction and the Side Products Formed.**

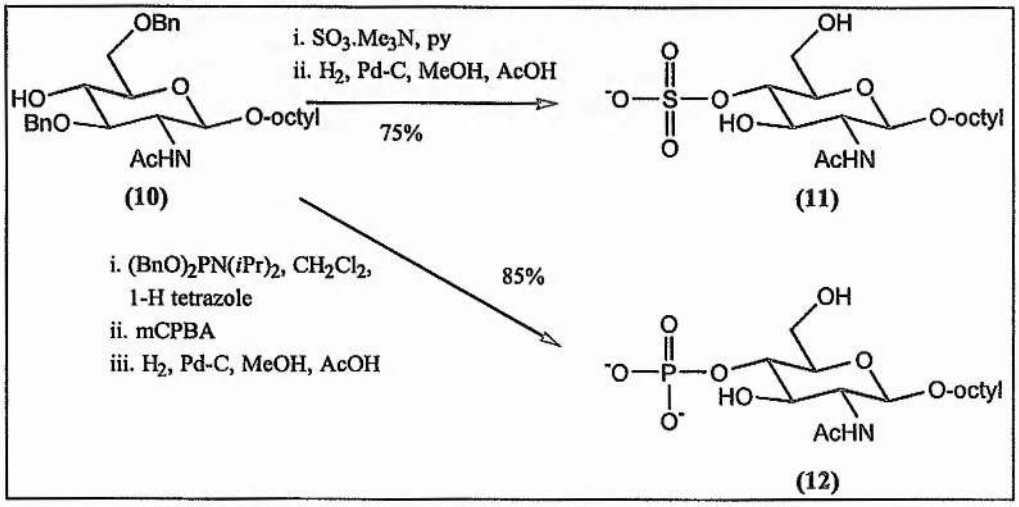
A recent publication by DeNinno and co-workers [8] showed an alternative route for selective cleavage of benzylidene acetal rings to give the same regiocontrol described for the reaction involving sodium cyanoborohydride. The paper reports the procedure to require less rigorously anhydrous conditions than the above mentioned procedure using sodium cyanoborohydride, and the yields quoted were typically 80-90% with no formation of the 6-alcohol (**10b**), or the 4,6-diol (**10a**) (**Scheme 3-10**). Following the work of DeNinno and co-workers, attempts were made to selectively cleave the benzylidene acetal of octyl 2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (**9**) using triethyl silane and trifluoroacetic acid in methylene chloride. Initial attempts to selectively cleave the benzylidene acetal showed the major product to be the 4,6-diol (**10a**). A reason for the formation of the diol (**10a**) could be due to trace amounts of water being present in the reaction flask. Aqueous acids are commonly used to cleave benzylidene acetals. Later attempts using anhydrous conditions were more successful, however the reaction was not selective as the desired 4-alcohol (**10**), the 4,6-diol, (**10a**), and the 6-alcohol, (**10b**), were isolated and identified by n.m.r. spectroscopy.

A more successful method for selective cleavage of the benzylidene acetal of compound (**9**) was using lithium aluminium chloride and borane trimethylamine complex

as reported by Vasella and co-workers [9]. According to Garegg and co-workers [7] in 1982 this method should give the 6-hydroxyl free leaving the benzyl ether at the 4-OH. However a later publication by the same author [10] reported that if tetrahydrofuran was used as a solvent rather than methylene chloride a reversal of regiochemical outcome occurred. This procedure was adopted and gave the desired product in reasonable to high yields with no observed formation of side products. However, disadvantages were the slow rate of reaction and the large quantities of the borane salt needed which had to be removed from the reaction mixture by column chromatography.

### 3.2.3 Synthesis of Sulfate and Phosphate Derivatives of Octyl GlcNAc

As a model study the sulfate and phosphate derivatives of octyl 2-acetamido-2-deoxy-β-D-glucopyranoside were prepared (**Scheme 3-11**). These may act as substrate or inhibitors for the enzyme bovine β-1,4-galactosyltransferase (**Chapter 4**).




**Scheme 3-11: The Synthesis of Sulfate and Phosphate Derivatives of Octyl GlcNAc[1]**

Sulfate (**11**) was prepared from alcohol (**10**) using sulfur trioxide trimethylamine complex and pyridine. Once the reaction was complete care was taken to leave the compound as the pyridinium salt as it was observed that complete removal of pyridine resulted in decomposition of the sulfonated compound. Accordingly removal of excess

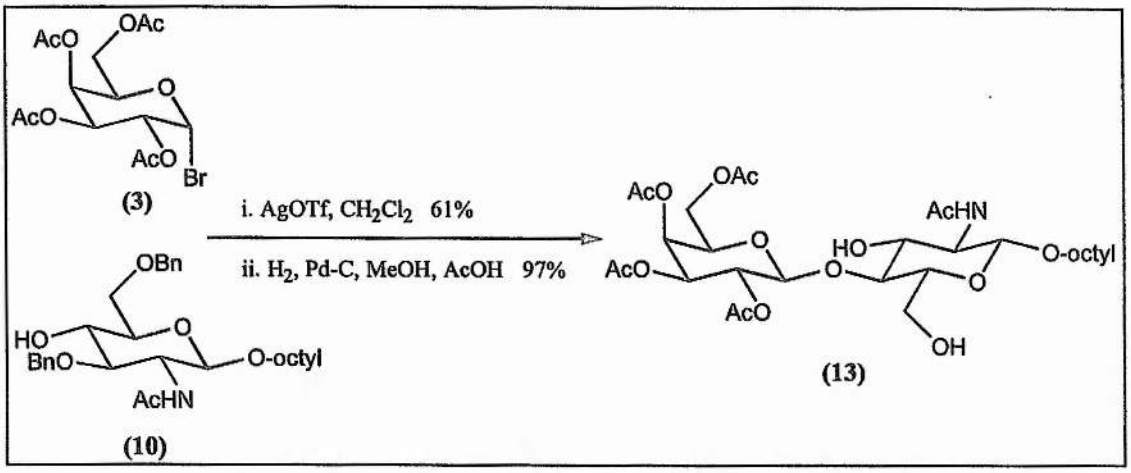
[1] Sulfonato and phosphonato derivatives were prepared as their sodium salts. For clarity the counter ion has been omitted from this and all subsequent diagrams.

sulfur trioxide trimethylamine complex from the reaction mixture was achieved by flash chromatography on silica gel using methylene chloride/methanol (10:1) with 0.2% pyridine. Directly prior to hydrogenation all the pyridine was removed from the mixture. Hydrogenation was monitored carefully and once the reaction was shown to be complete immediate work-up and purification of the compound was carried out. Purification was carried out by passing the sulfated compound through a short column of QAE-Sephadex AG-25 (anionic) and eluting with sodium chloride (1 M) directly onto a reverse phase $C_{18}$ SepPak cartridge. Elution from the SepPak yielded the sodium salt of the sulfated sugar (**11**). Once the sulfate was converted to the sodium salt the compound appeared to be relatively stable and could be stored indefinitely at -20 ° C.

Synthesis of phosphate (**12**) was achieved using phosphoramidite chemistry [11]. Alcohol (**10**) was treated with dibenzyldiisopropyl phosphoramidite in methylene chloride and once formation of the phosphite was complete the reaction mixture was subjected to *in situ* oxidation using *m*-chloroperbenzoic acid at -40 ° C. This reaction was high yielding however it was observed that anhydrous conditions were necessary for the reaction. The compound was deprotected by hydrogenation. Work up and purification was similar to that for compound (**11**). Initial attempts to phosphorylate compound (**10**) using tetrabenzylpyrophosphate [12] [13] and also diphenyl phosphorochloridate [14] were unsuccessful. This was though to be due to the low reactivity of the 4-OH of *N*-acetylglucosamine.

### 3.2.4 Synthesis of Sulfate and Phosphate Derivatives of *N*-Acetyllactosamine

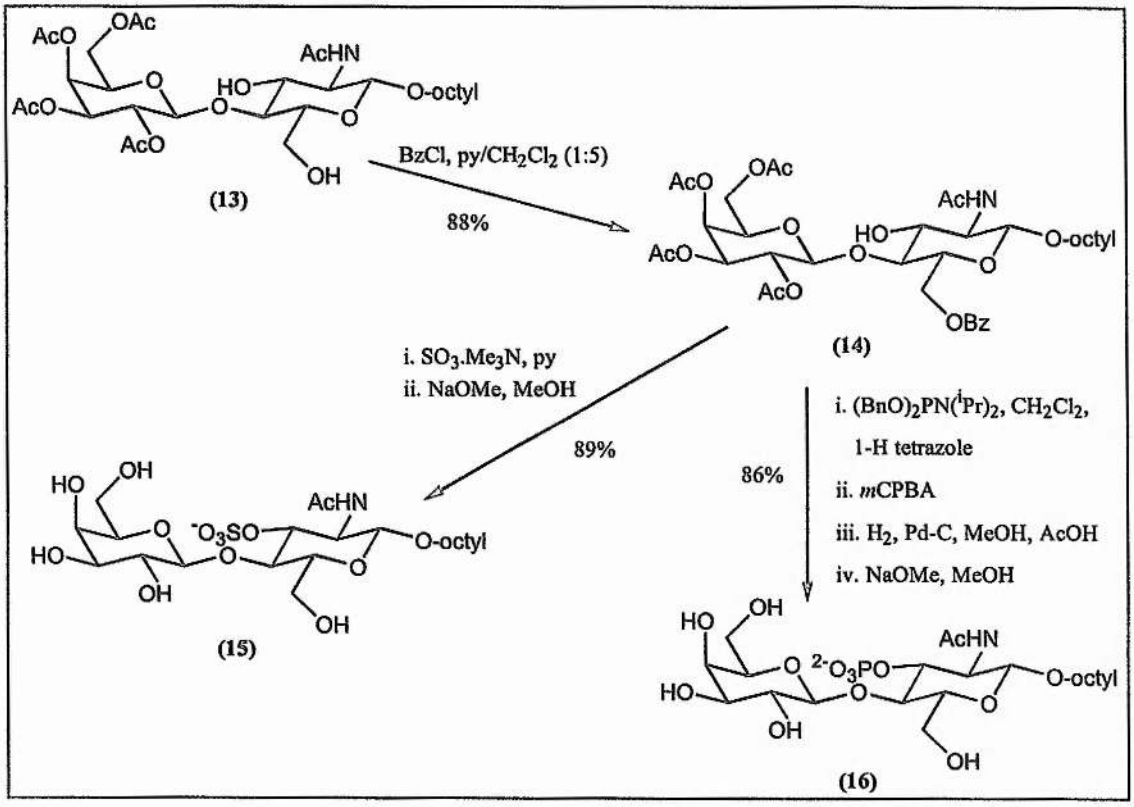
Chemical coupling using acetobromogalactose (**3**) as a glycosyl donor and 2-acetamido-3,6-di-*O*-benzyl-β-D-glucopyranoside (**10**) as a glycosyl acceptor was achieved using silver triflate [15] as a promoter (**Scheme 3-12**).




**Scheme 3-12: The Synthesis of Disaccharide (13)**

Monitoring the reaction by t.l.c. proved problematic due to the large quantities of degraded donor present in the reaction mixture. To determine the success of the reaction it was necessary to first deacteylate the complex mixture. Degraded donor was then removed by flash column chromatography and the mixture was re-acetylated. Diol (13) was then obtained by hydrogenation.

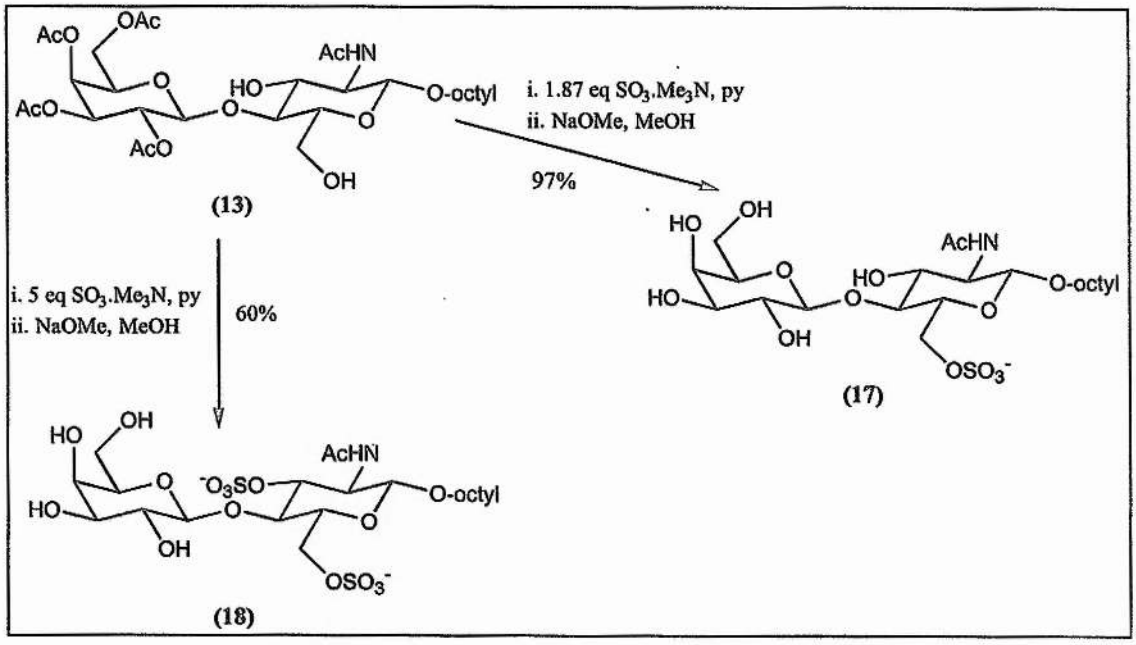
Synthesis of the 3-*O*-sulfonato (15) and 3-*O*-phosphonato (16) derivatives of octyl LacNAc were carried out as shown in Scheme 3-13.




**Scheme 3-13: Synthesis of 3-*O*-Sulfonato (15) and 3-*O*-Phosphonato (16) Derivatives of Octyl LacNAc**

The primary hydroxyl of diol (13) was selectively protected as a benzoate ester. Care had to be taken to prevent benzoylation of the 3-OH. Sulfation and phosphorylation of alcohol (14) were carried out as described for compounds (11) and (12) above, giving rise to (15) and (16) on deprotection.

Sulfation of the 6-OH of octyl LacNAc was achieved by a selective sulfation of compound (13) using less than 2 mole equivalents of sulfur trioxide trimethylamine complex. Deprotection using sodium methoxide yielded the 6-*O*-sulfonato derivative (17) (**Scheme 3-14**).



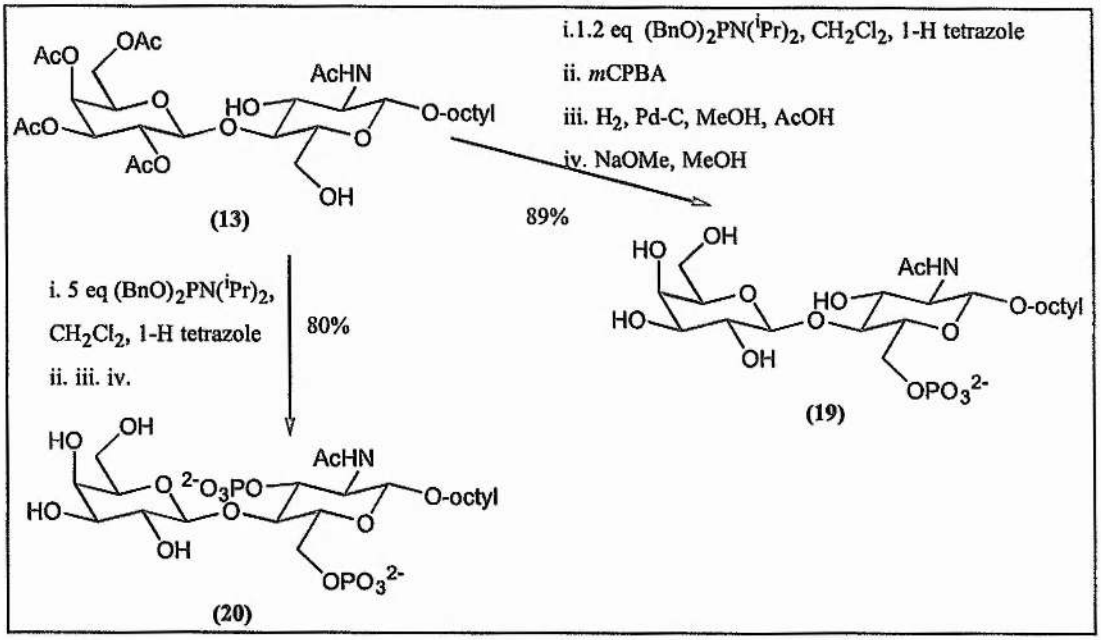

**Scheme 3-14: Synthesis of 6-*O*-Sulfonato (17) and 3,6-Di-*O*-Sulfonato (18) Derivatives of Octyl LacNAc**

Synthesis of the 3,6-di-*O*-sulfonato compound (18) was carried out using five mole equivalents of sulfur trioxide trimethylamine complex. However the reaction would not go to completion even at elevated temperatures and approximately 40% of the 3-*O*-sulfonato compound (17) was also obtained. Due to instability, separation of these compounds before deprotection was difficult by flash column chromatography. Separation of the mono- and di-sulfates was achieved on QAE-Sephadex A-25 using a gradient elution with NaCl (0.02-0.1 M).

Synthesis of the 6-*O*-phosphonato derivative (19) was carried out by selective phosphorylation of diol (13) using only 1.2 mole equivalents of dibenzyl diisopropyl phosphoramidite (**Scheme 3-15**). Oxidation, deprotection and purification was carried out as described for compound (12).

Di-*O*-phosphorylation of compound (13) was achieved using 5 mole equivalents of phosphitylating agent. The 3,6-di-*O*-phosphonato compound (20) was prepared in high yield with no trace of diol (13) or 6-*O*-phosphonato (19) remaining in the reaction (**Scheme 3-15**). During purification of the di-*O*-phosphonato compound it was observed that it had

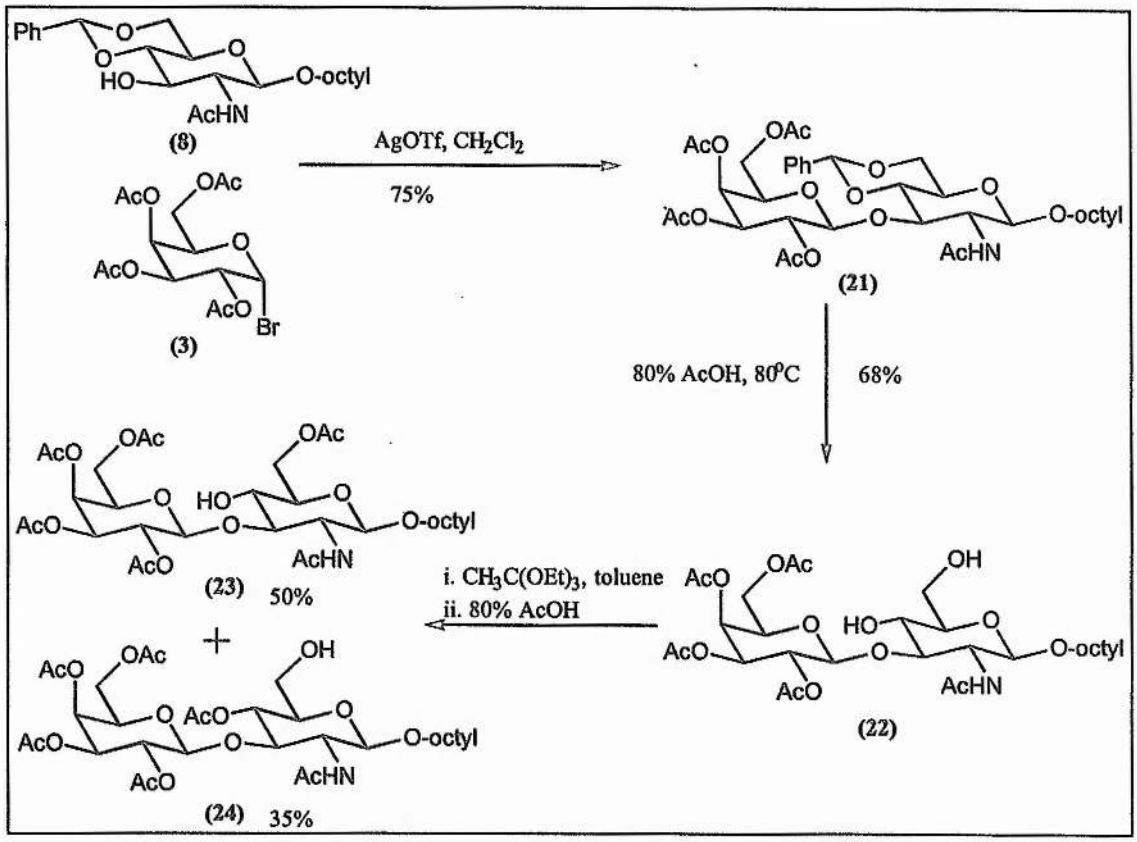
less affinity for the $C_{18}$ silica gel SepPak cartridges and care had to be taken to ensure the compound was not eluted from the cartridge during washing with water.




**Scheme 3-15: Synthesis of 6-*O*-Phosphonato (19) and 3,6-Di-*O*-Phosphonato (20) Derivatives of Octyl LacNAc**

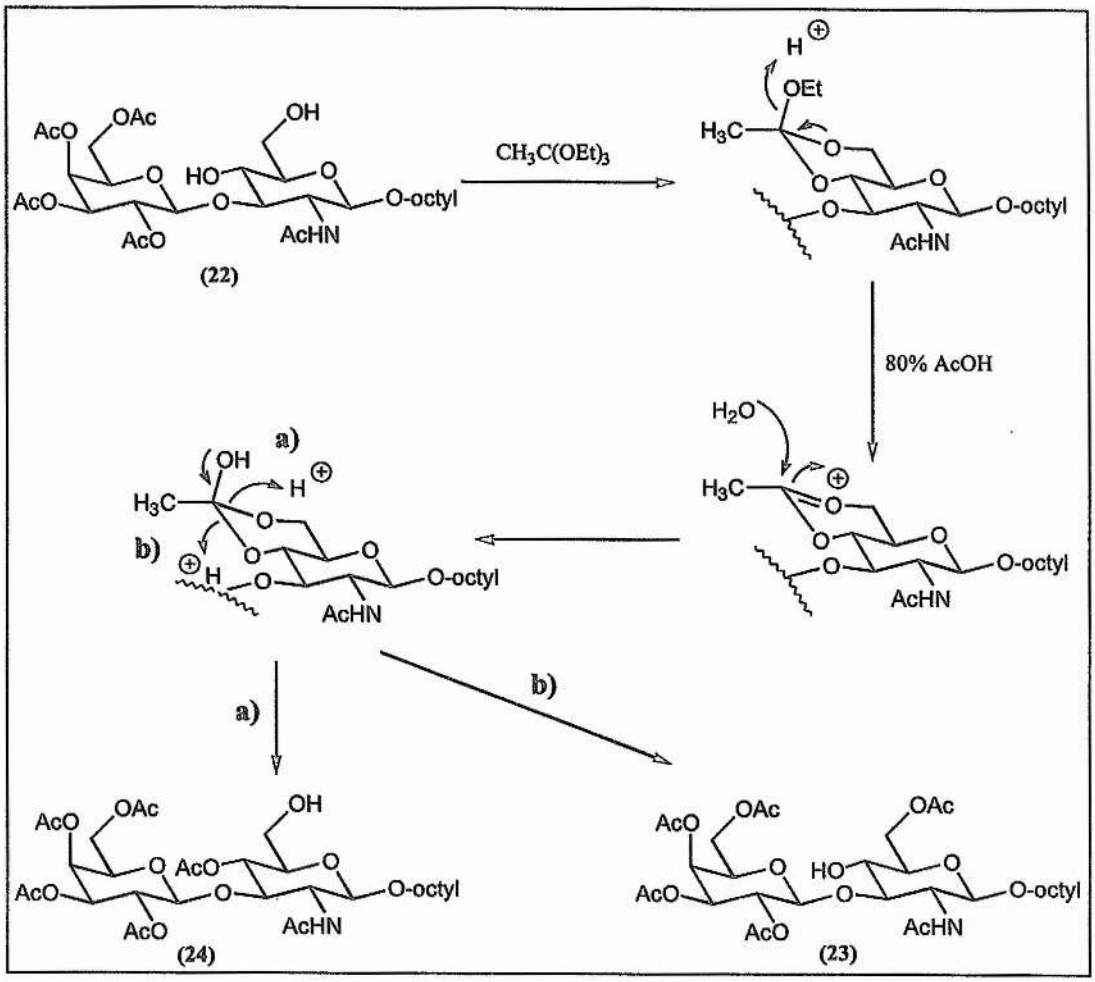
### 3.2.5 Synthesis of Sulfate and Phosphate Derivatives of Octyl 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside

Octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (8) was coupled with acetobromogalactose (3) using silver triflate as a promoter (**Scheme 3-16**). As for the synthesis of octyl LacNAc, a problem encountered was removal of the degraded donor from the reaction mixture. The reaction mixture was deacetylated, purified by column chromatography and re-acetylated to yield compound (21).



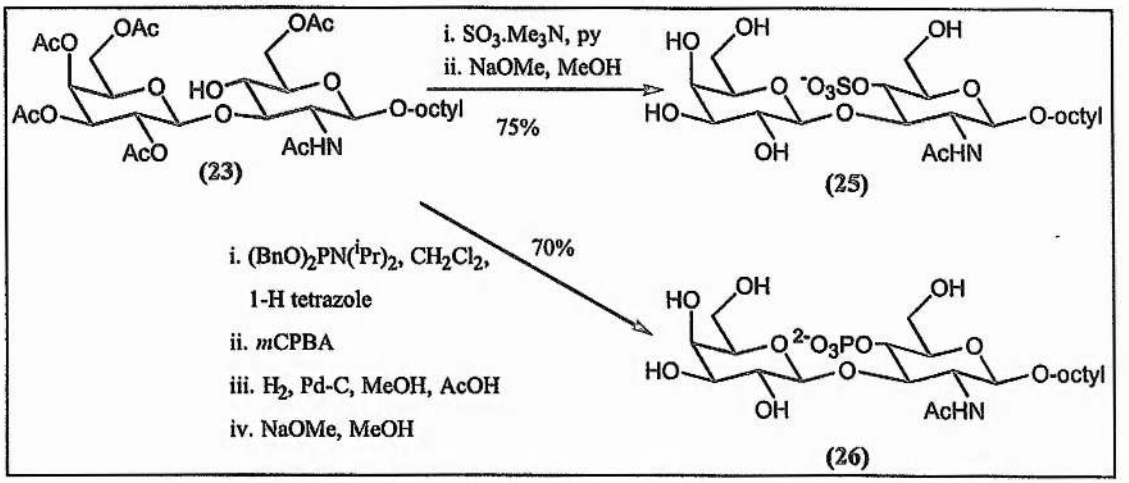

**Scheme 3-16: Synthesis of 6-*O*-Acetyl and 4-*O*-Acetyl Octyl Gal-β-1,3-GlcNAc (23) and (24)**

Compound (21) was heated in acetic acid for 2 hours to remove the benzylidene acetal to form diol (22). Temporary protection of the diol with an ortho ester [16] followed by treatment with acetic acid yielded a mixture of 6-*O*-acetate (23) and 4-*O*-acetate (24). It was observed that the major product from the reaction was the 6-*O*-acetate. This can be explained by considering the mechanism shown in **Scheme 3-17**. As for the mechanism for reductive opening of benzylidene acetals (**Scheme 3-9**), which product is favoured depends on the relative basicities of the 4- and 6-oxygen atoms.



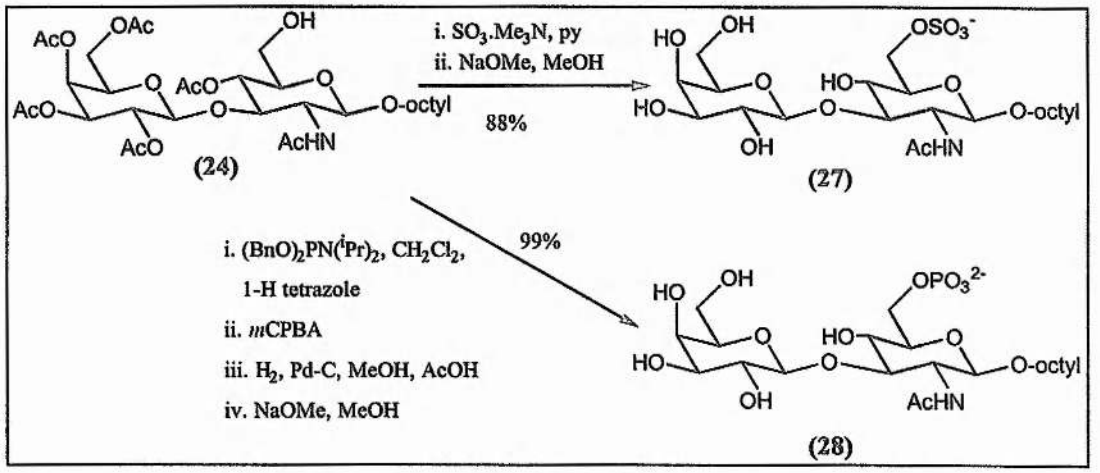

**Scheme 3-17: The Mechanism of Ortho Ester Cleavage**

Alcohol (23) was sulfated and phosphorylated as described for compounds (11) and (12) to yield compounds (25) and (26) respectively (Scheme 3-18).



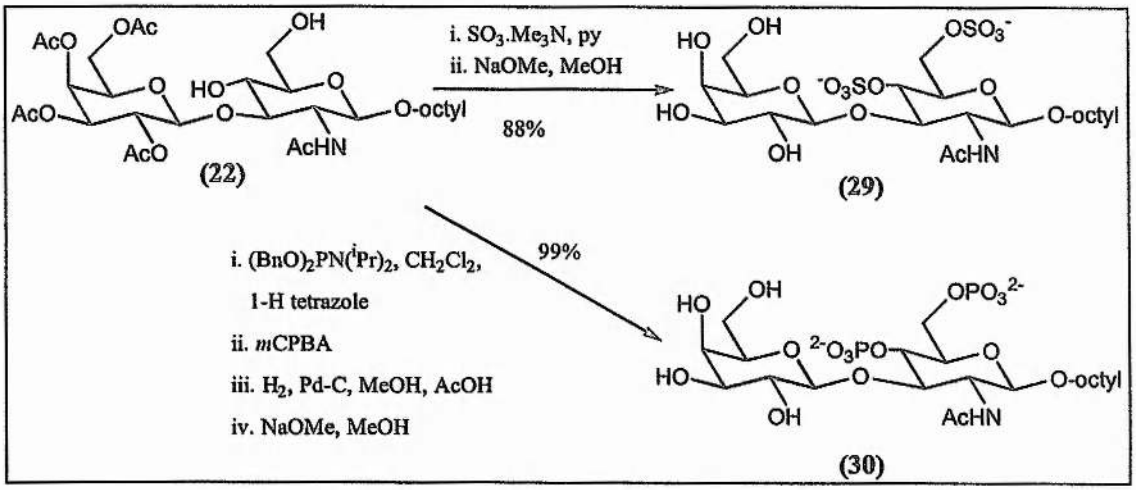

**Scheme 3-18: Synthesis of 4-*O*-Sulfonato (25) and 4-*O*-Phosphonato (26) Derivatives of Octyl Gal-β-1,3-GlcNAc**

Alcohol (24) was converted to its corresponding 6-*O*-sulfonato (27) and 6-*O*-phosphonato (28) derivatives (**Scheme 3-19**) as described for the conversion of (10) to (11) and (12).




**Scheme 3-19: Synthesis of 6-*O*-Sulfonato (27) and 6-*O*-Phosphonato (28) Derivatives of Octyl Gal-β-1,3-GlcNAc**

The 4,6-di-*O*-sulfonato (29) and phosphonato (30) derivatives of octyl Gal-β-1,3-GlcNAc were prepared from diol (22) as described for compounds (18) and (20) (**Scheme 3-20**).




**Scheme 3-20: Synthesis of 4,6-Di-*O*-Sulfonato (29) and 4,6-Di-*O*-Phosphonato (30) Derivatives of octyl Gal-β-1,3-GlcNAc**

### 3.2.6 Characterization of Sulfonato and Phosphonato Derivatives

Sulfated and phosphorylated sugars (15-30) were characterized by $^1H$ and $^{13}C$ n.m.r. spectroscopy and FAB or ES mass spectroscopy. Due to the nature of these compounds elemental analysis proved to be unsuccessful and on determining melting points, decomposition occurred. Optical rotations in all cases were less than 2° (c 0.1, MeOH). Octyl LacNAc (31) and octyl Gal-β-1,3-GlcNAc (32) were prepared by deacetylation of compounds (13) and (22) respectively using sodium methoxide in methanol.

Characteristic $^1H$ n.m.r. shift changes induced by substitution in the *N*-acetyllactosamine series of sulfates and phosphates are shown in **Figure 3.1**. An interesting observation is the down field shifts of the $H_1$. signal in the compounds containing a sulfate moiety at position 3 of the disaccharide. This is likely to be due to an overall change in orientation of the pyranose rings relative to one another induced by the steric effects of substitution at the site adjacent to the glycosidic linkage [6]. This torsional effect caused by sulfation at sites adjacent to glycosidic linkages has also been shown to occur for chondroitin sulfate related disaccharides [17]. A similar pattern can be observed for the

corresponding sugars containing a phosphonato moiety at position 3. Similarly **Figure 3.2** shows the $^{1}H$ n.m.r. data for all octyl Gal-β-1,3-GlcNAc sulfates and phosphates.

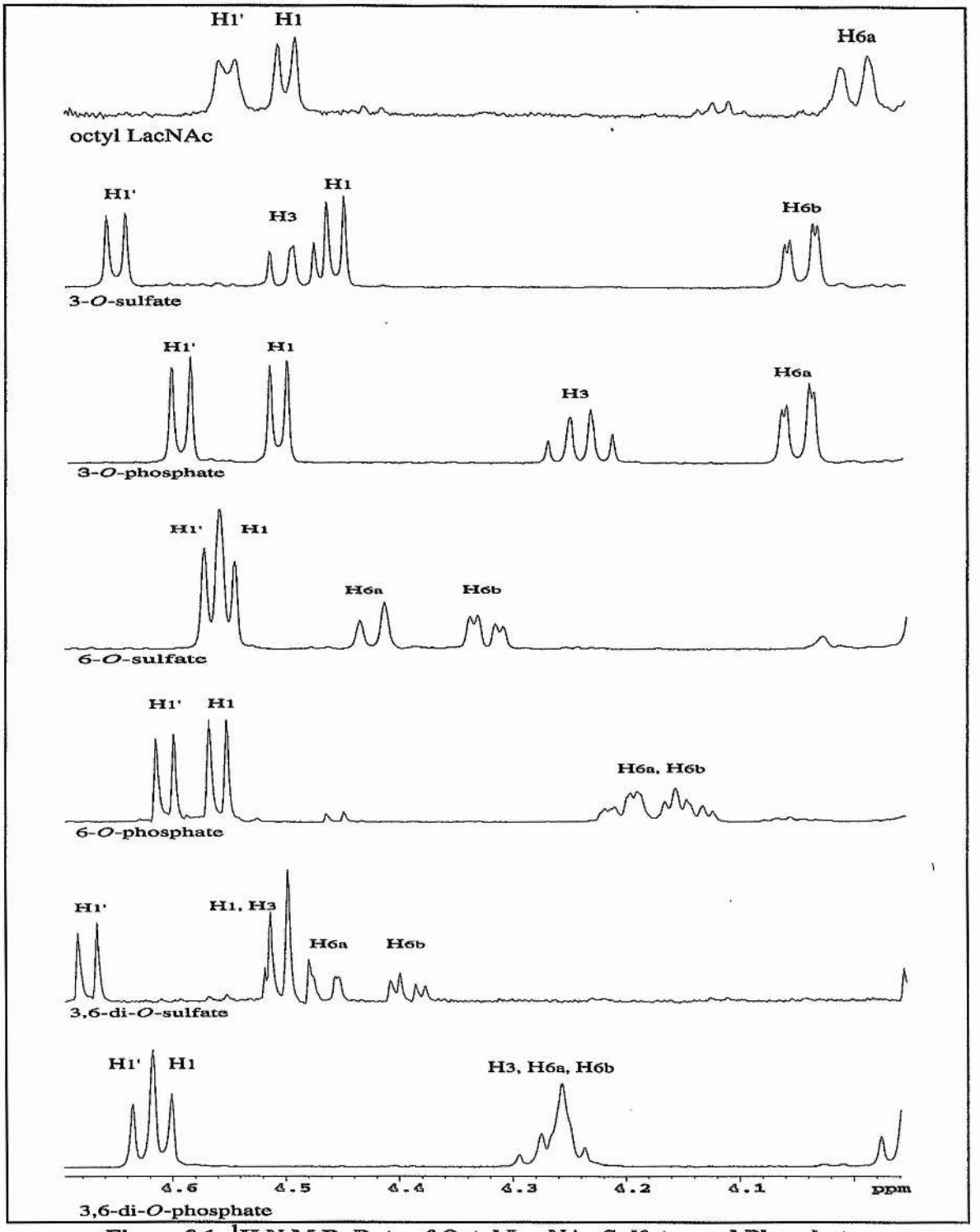



**Figure 3.1: $^{1}$H N.M.R. Data of Octyl LacNAc Sulfates and Phosphates**

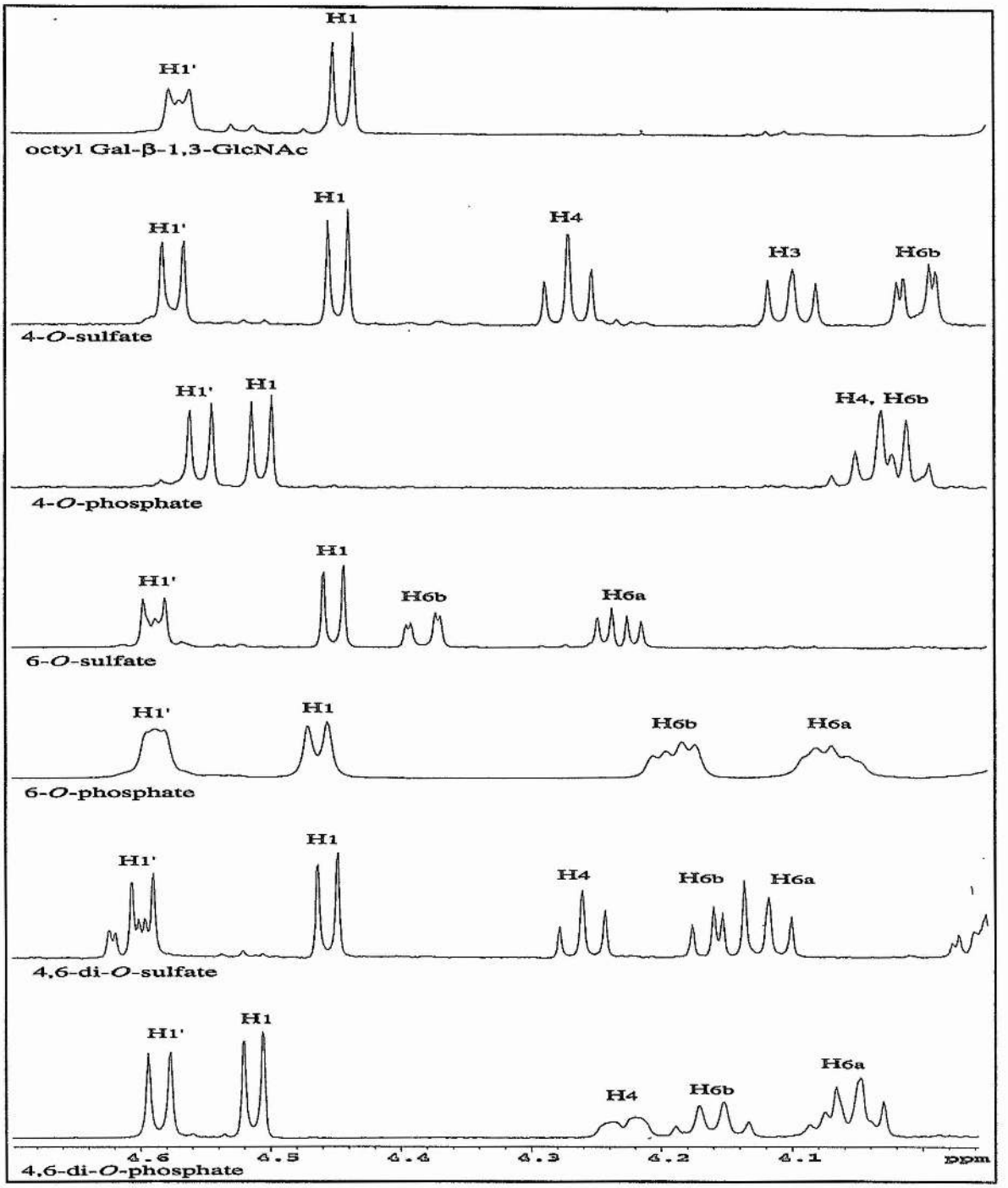
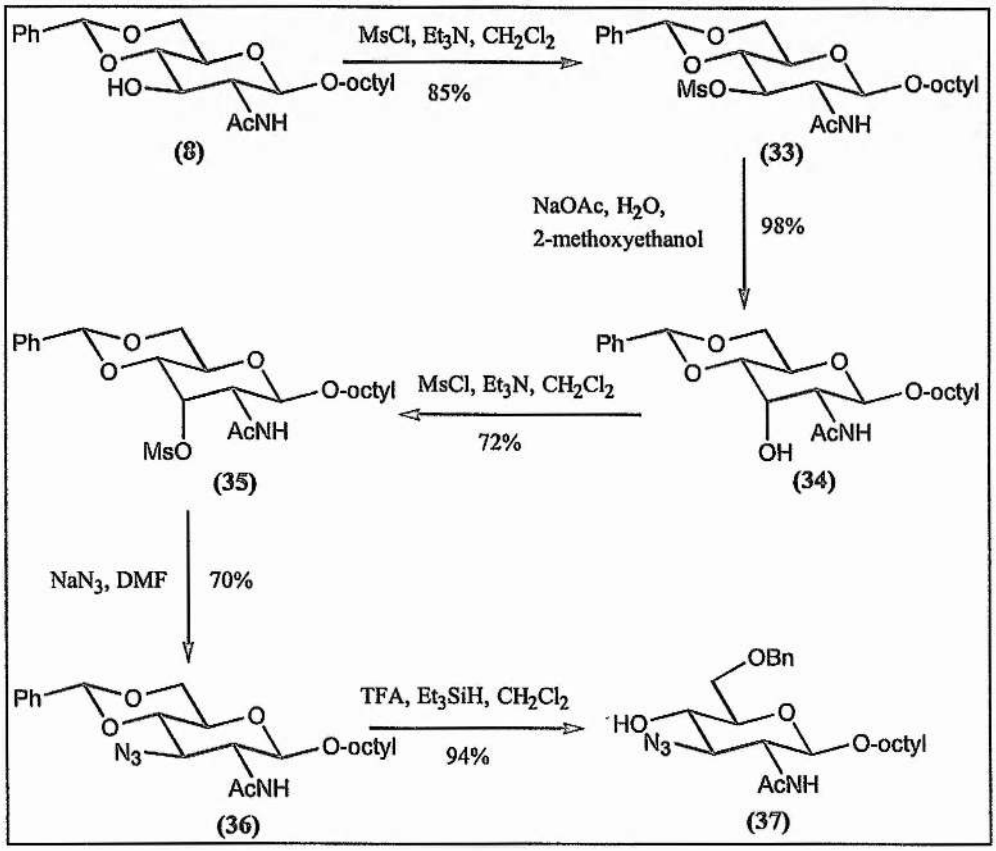



**Figure 3.2: $^{1}$H N.M.R. Data of Octyl Gal-β-1,3-GlcNAc Sulfates and Phosphates**

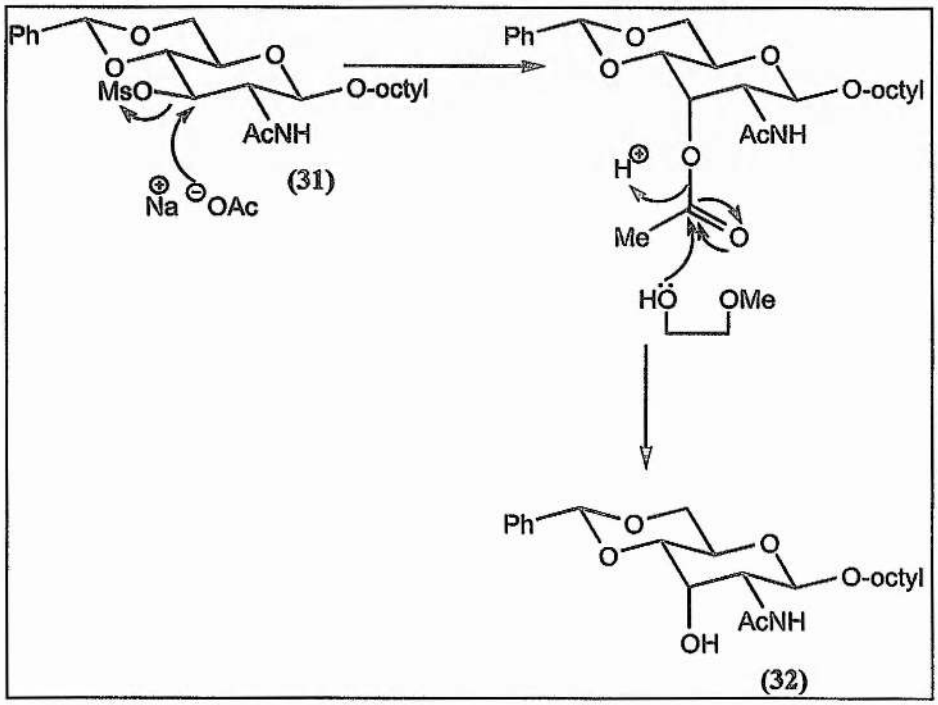
## *3.3 Attempted Synthesis of Octyl 2-Acetamido-3-amino-2-deoxy-4-O-(β-D-galactopyranosyl)-β-D-glucospyranoside (37)*

It has been shown that for some glycosyltransferases replacement of the hydroxyl to be glycosylated of the acceptor substrate with an amino group produces an inhibitor of the enzyme (**Chapter 1**). It is thought that rather than a hydrogen bond interaction with the active site base of the enzyme and the hydroxyl group of the acceptor, a charge-charge interaction results with the protonated form of the amino compound. To determine whether the 3-amino compound was an inhibitor of α-1,3-fucosyltransferases it was decided to prepare octyl 2-acetamido-3-amino-2-deoxy-4-O-(β-D-galactopyranosyl)-β-D-glucopyranoside (37). The synthesis of the acceptor substrate for chemical coupling is shown in **Scheme 3-21**.




**Scheme 3-21: Synthesis of Octyl 2-Acetamido-3-azido-2-deoxy-β-D-glucopyranoside (37)**

The alcohol (8) was sulfonylated using a procedure described by Vasella and co-workers [18] using methanesulfonyl chloride and triethylamine in methylene chloride (**Scheme 3-21**). The alcohol was insoluble in methylene chloride but dissolved on addition of the methanesulfonyl chloride. The reaction was complete within 0.5 h. Inversion of the stereochemistry at position 3 of octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulfonyl-β-D-glucopyranoside (33) was achieved using a method described by Jeanloz [19]. The sulfonylated compound (33), sodium acetate and water in 2-methoxyethanol were refluxed for 20 hours under argon. The mechanism for the reaction is shown in **Scheme 3-22** and first involves an $S_N2$ displacement of the methanesulfonate group by acetate anion. The hydroxyl group of 2-methoxyethanol attacks the acetate group to leave the unprotected alcohol octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-allopyranoside (34) in 98% yield.




**Scheme 3-22: Mechanism of Mesylate Displacement**

The *allo*-configured alcohol (34) was sulfonylated to give octyl 2-acetamido-3-*O*-methanesulfonyl-4,6-*O*-benzylidene-2-deoxy-β-D-allopyranoside (35) as described for the *gluco*-isomer. Azide (36) was obtained by heating (35) in DMF with sodium azide at 110°C

as described by Wong and co-workers [20]. Selective reduction of the benzylidene acetal of compound (36) was achieved using trifluoroacetic acid and triethylsilane in methylene chloride [8]. An interesting observation is the relatively high yield obtained for this reduction compared to that obtained for the same reaction with octyl 2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-β-D-glucopyranoside (10). The reductive ring opening of the 3-azido sugar proved to be high yielding and no side products were isolated from the reaction mixture. Perhaps for compound (10) the incoming electrophile is also coordinated by the benzyl group oxygen at the 3 position of the sugar and hence a competing reaction would be the formation of the 6-OH compound.

Attempts were made to chemically couple alcohol (37) with acetobromogalactose (3). However despite attempts at numerous different glycosylation methods (halide/AgOTf, halide/$HgBr_2$, imidate/$BF_3OEt_2$ and imidate/$I_2$) including those successfully used for chemical coupling of the 3,6-di-*O*-benzyl protected sugar (10) no formation of the desired compound was observed. This may be due to the steric bulk of the azido group at the site adjacent to the hydroxyl to be glycosylated.

## *3.4 References*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# *Chapter 4: Experimental*

## 4.1 General Details

t.l.c. was performed on silica gel 60 $GF_{254}$ (Merck) and compounds were detected with UV light or charring with dilute sulfuric acid or orcinol* as appropriate. Flash-column chromatography was performed on silica gel 60 (230-400 mesh, Merck). $^{1}H$, $^{13}C$ and $^{31}P$ n.m.r. spectra were recorded on a Varian Gemini 300 spectrometer (300 MHz, FT $^{1}H$ n.m.r.; 75 MHz, FT $^{13}C$ n.m.r.; 121 MHz, FT $^{31}P$ n.m.r.) usually using deuteriochloroform as the solvent and TMS as an internal standard for $^{1}H$ and $^{13}C$ spectra and usually using deuterium oxide as a solvent and phosphoric acid as an external reference for $^{31}P$ spectra. *J* values are given in Hz. M.p.s were determined on an Electrothermal melting point apparatus and are uncorrected. Elemental microanalyses were performed by the departmental microanalysis laboratory. Mass spectra were recorded by FAB-MS (using Clellands Matrix) or ES-MS as stated. I.R. spectra were recorded on a Perkin-Elmer 1710 FT IR spectrometer. Samples were typically prepared as a Nujol mull between sodium chloride discs. The frequency ($\nu$) of absorption maxima are given in wavenumbers ($cm^{-1}$) relative to a polystyrene standard. All the solvents used were either distilled or AnalaR quality. Solvents were dried according to literature procedures [1]. $CH_2Cl_2$ and toluene were distilled from $CaH_2$. THF was distilled from sodium/benzophenone under $N_2$. All solid reagents were dried over $P_2O_5$ overnight *in vacuo* prior to use. Sodium hydride was used as a 60% dispersion in oil, which was washed with hexane immediately before use. *m*CPBA was used at 50% purity. Anhydrous hydrogen chloride gas was generated by dripping concentrated sulfuric acid onto anhydrous NaCl in a two-neck round bottom flask. The gas generated was dried by passage through a Drechsel bottle containing calcium chloride. Reverse phase $C_{18}$ SepPak cartridges (0.5 $cm^3$ Waters, part no. WAT020515) were equilibrated before use by washing with $H_2O$ (10 $cm^3$), MeOH (10 $cm^3$) and $H_2O$ (10 $cm^3$). Extraction procedures were carried out by diluting with $CH_2Cl_2$ and washing sequentially with saturated aqueous $NaHCO_3$ solution and $H_2O$. For procedures using pyridine the solution in $CH_2Cl_2$ was first washed with 2M HCl. Organic extracts were dried using either anhydrous $Na_2SO_4$ or $MgSO_4$.

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* Orcinol spray was used for detecting low concentrations of deprotected sugars, and was prepared as follows: orcinol monohydrate (0.36g) was diluted in $H_2O$ (10$cm^3$). $H_2SO_4$ (20$cm^3$) and EtOH (150$cm^3$) were added.

*2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl bromide* (3).

To an ice-cold solution of D-galactose (10.96 g, 60.9 mmol) and DMAP in pyridine (20 $cm^3$) was added acetic anhydride (35 $cm^3$, 371 mmol) and the solution was stirred at r.t.. Once t.l.c. (toluene-EtOAc, 3:1) showed the reaction to be complete $CH_2Cl_2$ (150 $cm^3$) was added and the solution was subjected to a standard work-up to give galactopyranose pentaacetate as an α/β mixture which was used directly in the next step.

To a solution of galactopyranose pentaacetate (18.35 g, 470 mmol) and acetic anhydride (1 $cm^3$) in $CH_2Cl_2$ (50 $cm^3$) was added HBr (18 $cm^3$, 30% w/v solution in AcOH) and the solution was stirred for 2 h. The mixture was concentrated and AcOH was removed by co-evaporation with toluene to give an oil which was subjected to a standard work up. Flash chromatography of the resulting residue (toluene-EtOAc, 3:1) yielded an immobile oil which was crystallised to give the *title compound* as white needles (8.22 g, 43%); m.p. 83-85 °C ($Et_2O$), Lit. [2] 84-85 °C; $[\alpha]_D$ +200 (*c* 0.1, $CHCl_3$), Lit. $[\alpha]_D$ +217 (*c* 1, $CHCl_3$); $\delta_H$ ($CDCl_3$) 2.01 (3 H, s, O*Ac*), 2.06 (3 H, s, O*Ac*), 2.11 (3 H, s, O*Ac*), 2.15 (3 H, s, O*Ac*), 4.15 (2 H, m, 6-*H*, 6'-*H*), 4.48 (1 H, m,5-*H*), 5.05 (1 H, dd, $J_{2,3}$ 7.0 and $J_{3,4}$ 3.9 Hz, 3-*H*), 5.40 (1 *H*, dd, $J_{1,2}$ 3.5 Hz and $J_{2,3}$ 2-*H*), 5.52 (1 H, m, 4-*H*), 6.70 ( 1 H, d, $J_{1,2}$, 1-*H*).

*2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucosylpyranosyl chloride* (5)

Compound (5) was prepared using the procedure described by Horton [2]. Acetyl chloride (20 $cm^3$) was saturated with anhydrous HCl gas and added slowly to *N*-acetyl glucosamine (11.29 g, 51 mmoles). The suspension refluxed spontaneously during the first hour of the reaction. The solution was allowed to stir for 24 h until t.l.c. (toluene-EtOAc, 4:1) showed the reaction to be complete. The reaction mixture was diluted with $CH_2Cl_2$ (80 $cm^3$) and the solution was poured with stirring onto ice (80 g) and $H_2O$ (20 $cm^3$). The phases were separated and the organic phase was run off into iced saturated $NaHCO_3$ solution (80 $cm^3$). The organic phase was separated, washed with saturated $NaHCO_3$ solution, and run directly into a flask containing anhydrous $MgSO_4$. The solution was filtered and the filtrate was concentrated until only a small amount of $CH_2Cl_2$ was left (10 $cm^3$). $Et_2O$ (200 $cm^3$) was added and white needles of (5) formed spontaneously (12.65 g, 68%); m.p.125-127 °C Lit [2] 127-128 °C; $[\alpha]_D$ +105 (*c* 0.1, $CHCl_3$), Lit. [2] $[\alpha]_D$ +110 (*c* 1.1, $CHCl_3$) ; $\delta_H$ ($CDCl_3$), 1.99 (3H, s, NH*Ac*), 2.05 (6H, 2xs, O*Ac*), 2.10 (3H, s, O*Ac*),

4.21-4.33 (3 H, m, 5-H, 6-*H*, 6'-*H*), 4.53 (1 H, ddd, $J_{1,2}$ 3.7, $J_{2,3}$ 9.6, $J_{2,NH}$ 8.8 Hz, 2-*H*), 5.21 (1 H, t, *J* 9.5 Hz, 3-*H*/4-*H*), 5.33 (1 H, t, *J* 9.5 Hz, 3-*H*/4-*H*), 5.85 (1 H, br d, *J* 8.8 Hz, N*H*), 6.18 (1 H, d, $J_{1,2}$, 1-*H*).

*Octyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside* (**6**).

The title compound was prepared using a Koenigs-Knorr coupling procedure as described by Lemieux and co-workers [3]. To a suspension of $Hg(CN)_2$ (410 mg, 1.63 mmol), $HgBr_2$ (55 mg, 0.15 mmol) and anhydrous $CaSO_4$ (1.11 g) in anhydrous toluene (1 $cm^3$) was added octanol (0.5 $cm^3$, 2.84 mmoles). The flask was flushed with $N_2$ while stirring for 1 h. 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**5**) (507 mg, 1.39 mmoles) was added and the mixture was stirred at r.t. for 4 days. $CH_2Cl_2$ (50 $cm^3$) was added and solids were removed by filtration through Celite. The filtrate was subjected to a standard workup and purified by flash chromatography (silica gel; toluene-EtOAc, 1:1) affording compound (**6**) as a white solid (0.48 g, 75%); m.p. 125-128 °C; $[\alpha]_D$ -12.6 (*c* 0.1, $CHCl_3$); (Found C, 57.70; H, 8.24; N, 3.00. $C_{20}H_{37}O_9N$ requires C, 57.50; H, 8.11; N, 3.05.); $\delta_H$ ($CDCl_3$), 0.6-1.5 (15 H, 3 x m, octyl *H*), 1.95 (3 H, s, NH*Ac*), 2.03 (3 H, s, O*Ac*), 2.04 (3 H, s, O*Ac*), 2.09 (3 H, s, O*Ac*), 3.46 (1 H, m, -OC$H_2$ of octyl chain), 3.70 (1 H, m, 5-*H*), 3.83 (2 H, m, -OC$H_2$ of octyl, 2-*H*), 4.12 (1 H, dd, $J_{5,6}$ 2.5 and $J_{6,6'}$ 12.2 Hz, 6-*H*), 4.27 (1 H, dd, $J_{5,6'}$ 4.6 Hz and $J_{6,6'}$, 6'-*H*), 4.69 (1 H, d, $J_{1,2}$ 8.3 Hz, 1-*H*), 5.07 (1 H, t, *J* 9.8 Hz, 3-*H*/4-*H*), 5.32 (1 H, t, *J* 9.8 Hz, 3-*H*/4-*H*), 5.49 (1 H, br d, *J* 8.8 Hz, N*H*).

*Octyl 2-acetamido-2-deoxy-β-D-glucopyranoside* (**7**)

To a solution of octyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (**6**) (5 g, 11 mmoles) in MeOH (20 $cm^3$) was added a small piece of sodium and the solution was left to stand at r.t. for 4 h. Amberlite IR-120 ($H^+$) ion exchange resin (5 g) was added and the mixture was stirred for 5 mins. The resin was removed by filtration, washed with MeOH, and the filtrate was concentrated to dryness to give the *title compound* as an amorphous solid (3.48 g, 96%); $\delta_H$ ($CD_3OD$) 0.6-1.5 (15 H, 3 x m, octyl *H*), 1.98 (3 H, s, NH*Ac*), 3.31-4.26 (m, 2-6-*H*), 4.39 (1 H, d, $J_{1,2}$ 8.4 Hz, 1-*H*); FAB-MS: Calcd for $[C_{16}H_{31}NO_6]$ 333.1 obs: *m/z*: 356.0 [M + Na], 334.2 [M + 1].

*Octyl 2-acetamido-4,6-O-benzylidene-2-deoxy-β-D-glucopyranoside* (**8**)

To a solution of octyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**7**) (6.13 g, 18.4 mmoles) and *p*-TsOH (123 mg, 0.6 mmoles) in anhydrous $CH_3CN$ (120 $cm^3$) was added benzaldehyde dimethyl acetal (4.42 $cm^3$, 29.4 mmoles). Almost immediately the starting material started to dissolve and a white solid subsequently precipitated from the reaction mixture. The reaction was shown to be complete by t.l.c. (toluene-EtOAc, 2:1) after 10 mins. $Et_3N$ (0.075 $cm^3$, 2.5 eq with respect to *p*-TsOH) was added to quench the reaction. Water was added and the solid obtained was removed by filtration, washed with $H_2O$, and dried over $Na_2SO_4$. Crystallization gave the *title compound* as white needles (6.08 g, 78%); m.p. 218-220 °C (EtOH); $[\alpha]_D$ -56 (*c* 0.1, MeOH); (Found C, 65.88; H, 8.52; N, 3.28. $C_{23}H_{35}O_6N$ requires C, 65.53; H, 8.37; N, 3.32.); $\nu_{max}$ 3265 (OH), 2925 (NH), 1685 (CO); $\delta_H$ ($CDCl_3$); 0.6-1.5 (15 H, 3 x m, octyl *H*), 2.0 (3H, s, NH*Ac*), 3.3-3.6 (3 H, m), 3.7-4.0 (2 H, m), 4.1-4.4 (2 H, m), 4.72 (1 H, d, $J_{1,2}$ 8.2 Hz, 1-*H*), 5.56 (1 H, s, ArC*H*), 5.72 (1 H, br d, *J* 8.6 Hz, N*H*), 7.39 (5 H, m, *Ar*).

*Octyl 2-acetamido-3-O-benzyl-4,6-O-benzylidene-2-deoxy-β-D-glucopyranoside* (**9**)

Benzyl bromide (0.03 $cm^3$, 0.28 mmoles) was added to an ice cold solution of octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (**8**) (100 mg, 0.237 mmoles) in DMF (5 $cm^3$). Sodium hydride (3 mg, 0.3 eq) was added every 30 mins until t.l.c. (toluene-EtOAc, 2:1) showed the reaction to be complete. Ice-water was added to precipitate the product, which was collected by filtration and washed with $H_2O$. Flash chromatography (toluene-EtOAc, 2:1) gave the *title compound* as white needles (97 mg, 61%); m.p. 216-218 °C; $[\alpha]_D$ -4 (*c* 0.1, $CHCl_3$); (Found C, 70.97; H, 8.71; N, 2.76. $C_{30}H_{41}O_6N$ with 1 mol EtOH, C, 70.69; H, 8.71; N, 2.58.); $\nu_{max}$ 3265 (NH), 1655 (CO); $\delta_H$ ($CDCl_3$), 0.6-1.5 (15 H, 3 x m, octyl *H*), 1.87 (3 H, s, NH*Ac*), 3.2-3.9 (6 H, m), 4.34 (2 H, m), 4.65 (1 H, d, *J* 12.0 Hz, ArC$H_2$), 4.90 (1 H, d, *J* 12.0 Hz, ArC$H_2$), 5.00 (1 H, d, $J_{1,2}$ 8.3 Hz. 1-*H*), 5.56 (1 H, br d, *J* 8.0 Hz, N*H*), 5.58 (1 H, s, ArC*H*), 7.39 (10 H, m, *Ar*).

*Octyl 2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside* (**10**)

(a) Compound (**10**) was prepared using a selective acetal ring opening procedure described by Garegg and co-workers [4]. To an ice-cold suspension of octyl 2-acetamido-3-

O-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (**9**) (300 mg, 0.58 mmol), sodium cyanoborohydride (364 mg, 5.28 mmol) and powdered 4Å sieves and a crystal of methyl orange in THF was added a saturated solution of HCl in $Et_2O$ to acidify the reaction mixture. Once t.l.c. (toluene-EtOAc, 1:1) showed the reaction to be complete, ice-water was added and the solution was subjected to a standard work up. Flash chromatography (toluene-EtOAc, 2:1) gave a solid which was crystallised to give the *title compound* as white needles (170 mg, 57%); m.p. 116-118 °C (EtOAc/hexane); $[\alpha]_D$ -17 (*c* 0.1, $CHCl_3$); (Found C, 70.05; H 8.64; N, 2.70. $C_{30}H_{43}O_6N$ requires C, 70.15; H, 8.44; N, 2.73); $\delta_H$ ($CDCl_3$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.91 (3 H, s, NH*Ac*), 3.2-4.0 (6 H, m), 4.04 (1 H, dd, $J_{3,4}$ and $J_{4,5}$ 8.2 Hz, 3-*H*), 4.5-4.7 (7 H, m), 4.86 (1 H, d, $J_{1,2}$ 8.2 Hz, 1-*H*), 5.57 (1 H, br d, *J* 7.7 Hz, N*H*), 7.30 (10 H, br s, *Ar*).

b) Compound (**10**) was prepared using a selective acetal ring opening procedure described by DeNinno and co-workers [5]. To an ice-cold suspension of octyl 2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (**9**) (500 mg, 0.97 mmol) and triethylsilane (0.77 $cm^3$, 4.86 mmol) in $CH_2Cl_2$ (5 $cm^3$) was added TFA (0.37 $cm^3$, 4.86 mmol) dropwise. The solution was allowed to warm to r.t. Once t.l.c. (toluene-EtOAc, 1:1) showed the reaction to be complete (4 h), EtOAc was added and the solution was subjected to a standard work up. The product was purified by flash chromatography (toluene-EtOAc, 2:1) to afford the *alcohol* (**30**) as white needles (300 mg, 60%). Analytical data were identical to those obtained previously.

*Octyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-glucopyranoside* (**11**)

Alcohol (**10**) (200 mg, 0.4 mmol) was dissolved in pyridine (20 $cm^3$) and sulfur trioxide-trimethylamine complex (200 mg, 1.5 mmol) was added. The suspension was left to stir for 20 h. MeOH (1 $cm^3$) was added and the mixture was concentrated to dryness and purified by flash column chromatography ($CH_2Cl_2$ / MeOH 10:1 containing 0.2% v/v pyridine). The oil obtained was immediately dissolved in MeOH (20 $cm^3$) and palladium (5% on charcoal) (100 mg) was added. The suspension was acidified with AcOH and stirred under $H_2$ for 4 h. Filtration through Celite followed by concentration to dryness yielded the *title compound* as the free acid. Conversion to the sodium salt was achieved by passage through QAE-Sephadex A-25 ($OH^-$) and elution with NaCl solution (1M) (3x5 $cm^3$) directly

onto a $C_{18}$ SepPak. Washing with water (5 $cm^3$) followed by elution with MeOH (10 $cm^3$) and evaporation yielded the *title compound* as the sodium salt which was freeze dried from $H_2O$ (128 mg, 75%); $\delta_H$ ($D_2O$), 0.5-1.6 (15 H, 3xm, octyl *H*), 1.94 (3 H, s, NH*Ac*), 3.6-3.7 (2 H, m, 3-*H*, -OC$H_2$ of octyl), 3.8-3.9 (3 H, m, 5-*H*, 2-*H*, 6-*H*), 4.00 (1 H, m, -OC$H_2$ of octyl), 4.06 (1 H, dd, $J_{6,6'}$ 12.6 and $J_{5,6'}$ 2.3 Hz, 6'-*H*), 4.24 (1 H, t, $J_{3,4}$ 9.1 Hz, 4-*H*), 4.63 (1 H, d, $J_{1,2}$ 8.4 Hz, 1-*H*); $\delta_C$ ($D_2O$), 11.5, 20.1, 20.3, 23.2 (2xC), 26.4, 26.6, 29.2, 53.5, 58.7, 68.6, 70.4, 72.4, 75.2, 99.1, 172.5 (C=O); FAB-MS: Calcd for [$C_{16}H_{30}NO_9S$.Na], 430.21 obs: *m/z* 457.9 (M+Na).

*Octyl 2-acetamido-2-deoxy-4-O-phosphonato-β-D-glucopyranoside* (12)

To a mixture of alcohol (10) (140 mg, 0.27 mmol) and 1-*H* tetrazole (80 mg, 0.82 mmol) in $CH_2Cl_2$ (10 $cm^3$) was added *N,N*-diisopropyldibenzyl phosphoramidite (0.14 $cm^3$, 0.41 mmol). The reaction was stirred at r.t. until t.l.c. (toluene/EtOAc, 2:1) indicated the reaction to be complete (2 h). The mixture was cooled to -40 °C and *m*CPBA (90 mg, 0.54 mmol, 50% purity) in $CH_2Cl_2$ (5 $cm^3$) was added. The resulting solution was stirred at 0 °C for 0.5 h, diluted with $CH_2Cl_2$ (40 $cm^3$), washed with aqueous $Na_2SO_4$ (10%, 2x20 $cm^3$), saturated aqueous $NaHCO_3$ solution (2x15 $cm^3$) and saturated aqueous NaCl solution (20 $cm^3$). The organic extract was dried over $Na_2SO_4$, concentrated to dryness and purified by flash column chromatography (toluene/EtOAc, 2:1). The oil obtained was immediately dissolved in MeOH (20 $cm^3$) and palladium (5% on charcoal, 100 mg) was added. The suspension was acidified with AcOH and stirred under $H_2$ for 4 h. Filtration through Celite followed be concentration to dryness yielded the *title compound* as the free acid. Conversion to the sodium salt was achieved by passage through QAE-Sephadex A-25 ($OH^-$) and elution with NaCl solution (1M) (3x5 $cm^3$) directly onto a $C_{18}$ SepPak. Washing with water (5 $cm^3$) followed by elution with MeOH (10 $cm^3$) and concentration to dryness yielded the *title compound* as the sodium salt which was freeze dried from $H_2O$ (105 mg, 85%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 2.13 (3 H, s, NH*Ac*), 3.61 (1 H, m, 5-*H*), 3.69 (1 H, m, OC$H_2$), 3.8-3.9 (3 H, m, 4-*H*, 2-*H*, 6-*H*), 3.98-4.04 (2 H, m, 3-*H*, 6'-*H*), 4.61 (1 H, d, $J_{1,2}$ 8.2 Hz, 1-*H*); $\delta_C$ ($D_2O$) 13.3, 21.9, 22.1, 25.0, 28.3, 28.4, 28.5, 31.0, 55.3, 60.6, 70.5, 73.0, 73.7, 75.3, 101.2; $\delta_P$ ($D_2O$) 0.9; FAB-MS: Calcd for [$C_{16}H_{30}NO_9P.Na_2$] 457.0 obs: *m/z* 435.8 (M-Na), 457.8 (M).

*Octyl 2-acetamido-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-2-deoxy-β-D-glucopyranoside* (13)

A solution of alcohol (10) (750 mg, 1.5 mmol ) and bromide (3) (1.2 g, 2.9 mmol) in $CH_2Cl_2$ (20 $cm^3$) containing powdered 4Å sieves (2 g) was stirred under $N_2$ for 3 h and cooled to -30 °C. AgOTf (1.12 g, 4.4 mmol) was added and the suspension was stirred until t.l.c. ($CH_2Cl_2$/MeOH, 15:1) indicated the reaction to be complete. Collidine (0.9 $cm^3$, 5 mmol) was added and solids were removed by filtration through Celite. The filtrate was subjected to a standard workup, dried over $Na_2SO_4$ and concentrated to dryness. The residue obtained was dissolved in MeOH (25 $cm^3$) containing palladium (5% on charcoal, 150 mg) and subjected to hydrogenation until t.l.c. (toluene/EtOAc, 1:1) indicated the reaction to be complete (5 h). The suspension was filtered through Celite and the filtrate was concentrated to dryness. Flash chromatography (toluene/EtOAc, 1:1 → 0:1) yielded the *title compound* as a white solid (610 mg, 63%); m.p. 140-142 °C; $[\alpha]_D$ <2 (*c* 0.1, $CHCl_3$); (Found C, 53.97; H, 7.61; N, 2.22. $C_{30}H_{49}O_{15}N$ requires C, 54.29; H, 7.44; N, 2.11.); $\delta_H$ ($CDCl_3$), 0.6-1.5 (15 H, m, octyl *H*), 1.92 (3 H, s, NH*Ac*), 1.93 (3 H, s, O*Ac*), 2.00 (3 H, s, O*Ac*), 2.04 (3 H, s, *OAc*), 2.90 (3 H, s, O*Ac*), 3.3-3.4 (3 H, m), 3.5-3.6 (2 H, m), 3.7-3.8 (2 H, m), 3.9-4.2 (4 H, m), 4.63 (1 H, d, $J_{1,2}$ 7.9 Hz, 1-*H*), 4.71 (1 H, d, $J_{1',2'}$ 7.9 Hz, 1'-*H*), 4.98 (1 H, dd, $J_{2',3'}$ 10.4 and $J_{3',4'}$ 3.7 Hz, 3'-*H*), 5.15 (1 H, dd, $J_{2',3'}$ and $J_{2',1'}$, 2'-*H*), 5.32 (1 H, m, 4'-*H*), 5.57 (1 H, br d, 7.5 Hz, N*H*).

*Octyl 2-acetamido-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-6-O-benzoyl-2-deoxy -β-D glucopyranoside* (14).

Diol (13) ( 250 mg, 0.38 μmol ) was dissolved in pyridine-$CH_2Cl_2$ (1:5 v/v, 8 $cm^3$) and cooled on an ice bath. BzCl (0.05 $cm^3$, 0.47 μmol) was added and the mixture was allowed to warm to r.t. Once t.l.c. (EtOAc) indicated the reaction to be complete (3 h) MeOH was added and the solution was concentrated to dryness. Silica gel chromatography (toluene → toluene/EtOAc 1:1) gave (14) as a colourless solid (255 mg, 88%); $\delta_H$ ($CDCl_3$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.89 (3 H, s, O*Ac*), 1.94 (3 H, s, O*Ac*), 2.00 (3 H, s, O*Ac*), 2.09 (3 H, s, NH*Ac*), 3.2-3.6 (3 H, m), 3.6-3.8 (3 H, m), 3.8-4.1 (3 H, m), 4.23 (1 H, dd, $J_{6a,b}$ 12 and $J_{5,6b}$ 5.4 Hz, 6a-*H*), 4.53 (1 H, d, $J_{1,2}$ 7.8 Hz, 1-*H*), 4.75 (1 H, d, $J_{1',2'}$ 8.1 Hz, 1'-*H*),

4.88 (1 H, dd, $J_{3',4'}$ 3.3 and $J_{2',3'}$ 10.5 Hz, 3'-*H*), 5.17 (1 H, dd, $J_{2',3'}$ and $J_{1',2'}$, 2'-*H*), 5.30 (1 H, d, $J_{3',4'}$, 4'-*H*), 5.61 (1 H, d, *J* 8.2 Hz, N*H*), 7.2-7.4 (3 H, m, *Ar*), 7.96 (2 H, m, *Ar*).

*Octyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-3-O-sulfonato-β-D glucopyranoside* (**15**).

Sulfation was carried out as described for compound (**11**) using alcohol (**14**) (130 mg, 0.17 mmol), pyridine (10 $cm^3$) and sulfur trioxide-trimethylamine complex (68 mg, 0.49 mmol). The oil obtained was dissolved in MeOH (20 $cm^3$) and NaOMe (30 mg) in MeOH (5 $cm^3$) was added. The reaction was stirred at r.t. for 4 h and Amberlite IR-120 ($H^+$) was added until neutralisation had occurred. Concentration to dryness and purification of the desired product was carried out as described for compound (**11**), yielding (**15**) as a white solid (90 mg, 89%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, m, octyl *H*), 1.88 (3 H, s, NH*Ac*), 3.3-3.9 (11 H, m), 4.05 (1 H, dd, $J_{6a,b}$ 10.3 and $J_{5,6a}$ 2.1 Hz, 6a-*H*), 4.45 (1 H, d, $J_{1,2}$ 6.8 Hz, 1-*H*), 4.49 (1 H, dd, $J_{2,3}$ 10.3 and $J_{3,4}$ 8.9 Hz, 3-*H*), 4.65 (1 H, d, $J_{1',2'}$ 8.2 Hz, 1'-*H*); $\delta_C$ ($D_2O$), 13.3, 22.0, 22.4, 25.0, 28.3, 28.5 (2xC), 31.1, 54.7, 60.0, 61.0, 68.9, 70.7, 71.1, 72.9, 75.1 (2xC), 75.9, 79.3, 100.5, 103.3; FAB-MS: Calcd for [$C_{22}H_{40}NO_{14}S.Na$] 597.61 obs: *m/z* 619.8 (M+Na).

*Octyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-3-O-phosphonato-β-D glucopyranoside* (**16**).

Phosphorylation of alcohol (**14**) was carried out as described for compound (**12**) using alcohol (**14**) (100 mg, 0.13 mmol), 1-*H* tetrazole (30 mg, 0.39 mmol), *N,N*-diisopropyldibenzyl phosphoramidite (0.065 $cm^3$, 0.19 mmol) and *m*CPBA (50 mg, 0.26 mmol). The oil obtained was dissolved in MeOH (20 $cm^3$) and palladium (5% on charcoal, 100 mg) was added. The suspension was acidified with AcOH and stirred under $H_2$ for 4 h. The reaction mixture was filtered through Celite, concentrated to dryness and dissolved in MeOH (20 $cm^3$). NaOMe (50mg) in MeOH (5 $cm^3$) was added and the solution was left to stir at r.t. for 4 h. Amberlite IR-120 ($H^+$) was added until neutralisation had occurred. Evaporation and purification of the desired product was carried out as described for compound (**11**) yielding (**16**) as a white solid (69 mg, 86%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.91 (3 H, s, NH*Ac*), 3.4-3.8 (11 H, m), 4.05 (1 H, dd, $J_{6a,b}$ 12 and $J_{5,6a}$ 2.3 Hz,

6a-*H*), 4.24 (1 H, dd, $J_{3,4}$ 9.8 and $J_{2,3}$ 10.4 Hz, 3-*H*), 4.51 (1 H, d, $J_{1,2}$ 7.5 Hz, 1-*H*), 4.60 (1 H, d, $J_{1',2'}$ 8.1 Hz, 1'-*H*); $\delta_C$ ($D_2O$), 13.4, 22.0, 22.4, 25.0, 28.3, 28.5 (2xC), 31.1, 55.2, 60.0, 60.9, 68.7, 70.6, 70.9, 72.6, 75.1, 75.6, 76.1, 77.4, 100.8, 103.5; $\delta_P$ ($D_2O$) 3.2; FAB-MS: Calcd for [$C_{22}H_{41}NO_{14}P.Na_2$] 619.5 obs: *m/z* 620.2 (M+1).

*Octyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-6-O-sulfonato-β-D glucopyranoside* (17).

Sulfation of 6-OH of diol (13) was carried out as described for sulfate (11) using diol (13) (50 mg, 0.07 mmol), pyridine (2 $cm^3$) and sulfur trioxide-trimethylamine complex (20 mg, 0.14 mmol). Deprotection and purification of sulfate (17) was carried out as described for sulfate (15). The *title compound* was obtained as a white solid (44 mg, 97%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.91 (3 H, s, NH*Ac*), 3.35-3.90 (12 H, m), 4.32 (1 H, dd, $J_{6a,b}$ 11.3 and $J_{5,6b}$ 4.1 Hz, 6b-*H*), 4.43 (1 H, dd, $J_{6a,b}$ and $J_{5,6a}$ 1.2 Hz, 6a-*H*), 4.57 (1 H, d, $J_{1,2}$ 6.4 Hz, 1-*H*), 4.59 (1 H, d, $J_{1',2'}$ 5.8 Hz, 1'-*H*); $\delta_C$ ($D_2O$), 13.4, 22.0, 22.2, 25.04, 28.3, 28.5 (2xC), 31.1, 55.2, 61.1, 66.4, 68.7, 70.7, 71.1, 72.4, 72.6, 75.4, 77.6, 101.2, 102.6, 174.6 (C=O); ES-MS: Calcd for [$C_{22}H_{41}NO_{14}S$] 574.61 obs: *m/z* 573.7 (M-1).

*Octyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-3,6-di-O-sulfonato-β-D glucopyranoside* (18).

Sulfation of 6-OH of diol (13) was carried out as described for sulfate (11) using diol (13) (70 mg, 0.1 mmol), pyridine (5 $cm^3$) and sulfur trioxide-trimethylamine complex (120 mg, 0.84 mmol). Deprotection and purification of the residue obtained was carried out as described for sulfate (15). The *title compound* was obtained as a white solid (43 mg, 60%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, m, octyl *H*), 2.01 (3 H, s, NH*Ac*), 3.5-3.8 (5 H, m), 3.8-4.0 (5 H, m), 4.43 (1 H, dd, $J_{6a,b}$ 11.2 and $J_{5,6a}$ 4.3 Hz, 6a-*H*), 4.50 (3 H, m, 6b-*H*, 3-*H*, 1-*H*), 4.68 (1 H, d, $J_{1',2'}$ 8.7 Hz, 1'-*H*); ES-MS: Calcd for [$C_{22}H_{41}NO_{17}S_2$] 654.67 obs: *m/z* 327.4 ($M^{2-}$).

*Octyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-6-O-phosphonato-β-D glucopyranoside* (19).

Phosphorylation of the 6-OH of diol (13) was carried out as described for phosphate (12), using alcohol (13) (50 mg, 0.07 mmol), 1-*H* tetrazole (13 mg, 0.18 mmol), *N,N*-diisopropyldibenzyl phosphoramidite (0.03 $cm^3$, 0.02 mmol) and *m*CPBA (25 mg, 0.15 mmol). Deprotection and purification of the residue obtained was carried out as described for compound (16) The *title compound* was obtained as a white solid (41 mg, 89%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, m, octyl *H*), 1.91 (3 H, s, NH*Ac*), 3.3-3.9 (12 H, m), 4.15 (1 H, dd, $J_{6a,b}$ 11.5 and $J_{5,6b}$ 4.0 Hz, 6b-*H*), 4.22 (1 H, dd, $J_{6a,b}$ and $J_{5,6a}$ 2.9 Hz, 6a-*H*), 4.56 (1 H, d, $J_{1,2}$ 8.0 Hz, 1-*H*), 4.60 (1 H, d, $J_{1',2'}$ 7.5 Hz, 1'-*H*); $\delta_C$ ($D_2O$), 13.4, 22.0, 22.2, 25.0, 28.3, 28.5 (2xC), 31.1, 55.2, 61.0, 63.2, 68.7, 70.7, 71.2 (2xC), 72.6, 75.3, 77.9, 101.2, 102.7; $\delta_P$ ($D_2O$), 3.5; ES-MS: Calcd for [$C_{22}H_{42}NO_{14}P$] 574.53 obs: *m/z* 573.4 (M-1).

*Octyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-3,6-di-O-phosphonato-β-D glucopyranoside* (20).

Phosphorylation, deprotection and purification of diol (13) were carried out as described for compound (16) using alcohol (13) (70 mg, 0.11 mmol), 1-*H* tetrazole (44 mg, 0.63 mmol), *N,N*-diisopropyldibenzyl phosphoramidite (0.11 $cm^3$, 0.32 mmol) and *m*CPBA (70 mg, 0.42 mmol). The *title compound* was obtained as a white solid (65 mg, 80%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 2.05 (3 H, s, NH*Ac*), 3.5-3.66 (2 H, m), 3.68-3.80 (3 H, m), 3.80-4.0 (5 H, m), 4.26 (2 H, m, 6b-*H*, 4-*H*), 4.62 (2 H, dd, $J_{1,2}$ and $J_{1',2'}$ 8.3 Hz, 1-*H*, 1'-*H*); $\delta_C$ ($D_2O$), 10.1, 18.7, 19.1, 21.8, 25.1, 25.2, 27.8, 51.9, 57.6, 59.9, 65.6, 67.5, 67.9, 69.4, 70.4, 72.3, 73.1, 73.9, 86.9, 97.6, 100.1; $\delta_P$ ($D_2O$), 3.0, 4.1; ES-MS: Calcd for [$C_{22}H_{43}NO_{17}P_2$] 655.09 obs: *m/z* 327.2 ($M^{2-}$).

*Octyl 2-acetamido-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-4,6-O-benzylidene-2-deoxy-β-D glucopyranoside (21).*

Octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (8) (1.5 g, 3.6 mmol) and bromide (3) (2.93 g, 7.1 mmol) in $CH_2Cl_2$ (30 $cm^3$) containing powdered 4Å sieves (3 g) was stirred under $N_2$ for 3 h and cooled to -30 °C. AgOTf (2.83 g, 11 mmol)

was added and the suspension was stirred until t.l.c. (toluene/EtOAc, 1:1) indicated the reaction to be complete. Collidine (2.18 $cm^3$, 16 mmol) was added and the solids were removed by filtration through Celite. The filtrate was subjected to a standard workup, dried over $Na_2SO_4$ and concentrated. The reaction mixture was dissolved in MeOH (20 $cm^3$) and NaOMe (30 mg) was added. The solution was stirred at r.t. for 2 h and Amberlite IR-120 ($H^+$) was added until neutralisation had occurred. The mixture was filtered and the filtrate concentrated to dryness. Flash column chromatography (silica gel; EtOAc/EtOH/$H_2O$ 6:2:1) gave a crunchy solid to which pyrididne (15 $cm^3$) and $Ac_2O$ (12 $cm^3$) were added and the solution left to stir o/n. Azeotropic distillation with toluene followed by crystallisation (EtOH) yielded the *title compound* as a white solid (2.02 g, 75%); $\delta_H$ ($CDCl_3$) 0.6-1.5 (15 H, 3xm, octyl *H*), 1.89 (3 H, s, O*Ac*), 1.91 (3 H, s, O*Ac*), 1.93 (3 H, s, O*Ac*), 1.98 (3 H, s, O*Ac*), 2.01 (3 H, s, NH*Ac*), 3.3-3.54 (3 H, m), 3.56-3.9 (4 H, m), 3.98 (1 H, dd, $J_{6a,b}$ 11.2 and $J_{5,6a}$ 3.5 Hz, 6a-*H*), 4.26 (1 H, dd, $J_{6a,b}$ and $J_{5,6b}$ 4.5 Hz, 6b-*H*), 4.65 (1 H, d, $J_{1,2}$ 8.6 Hz, 1-*H*), 4.66 (1 H, d, $J_{1',2'}$ 8.8 Hz, 1'-*H*), 4.85 (1 H, dd, $J_{3',4'}$ 3.5 and $J_{2',3'}$ 10.1 Hz, 3'-*H*), 5.4-5.56 (2 H, m), 5.22 (1 H, d, $J_{3',4'}$, 4'-*H*), 5.44 (1 H, s, ArC*H*), 5.72 (1 H, br d, *J* 8.4 Hz, N*H*), 7.2-7.4 (5 H, m, Ar).

*Octyl 2-acetamido-6-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D glucopyranoside (23) and octyl 2-acetamido-4-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D glucopyranoside (24)*

Disaccharide (21) (1 g, 1.33 mmol) was dissolved in AcOH (80%) and heated at 80°C for 2 h. Azeotropic distillation with toluene followed by flash column chromatography (EtOAc) yielded the (22) as a white solid (600 mg, 68%) which was used directly in the next step.

Diol (22) (600 mg, 0.9 mmol) was dissolved in toluene (30 $cm^3$) and triethylorthoacetate (1.66 $cm^3$, 9 mmol) and *p*-TsOH (catalytic) were added. The reaction was stirred at r.t. for 45 mins, neutralised with $Et_3N$ and diluted with $CHCl_3$. The organic extract was washed with $H_2O$, dried over $Na_2SO_4$ and concentrated to dryness. The residue obtained was dissolved in AcOH (80%) and heated at 40°C for 10 mins. Azeotropic distillation with toluene followed by flash chromatography (toluene/EtOAc, 1:1) yielded acetates (23) (330 mg, 50%); $\delta_H$ ($CDCl_3$) 0.6-1.5 (15 H, 3xm, octyl *H*), 1.97 (3 H, s, O*Ac*),

1.99 (3 H, s, O*Ac*), 2.05 (3 H, s, O*Ac*), 2.07 (3 H, s, O*Ac*), 2.08 (3 H, s, O*Ac*), 2.14 (3 H, s, NH*Ac*), 2.96 (1 H, m, -OC$H_2$ of octyl), 3.47 (5 H, m), 3.7-4.5 (11 H, m), 4.56 (1 H, d, $J_{1,2}$ 8.1 Hz, 1-H), 4.92 (1 H, d, $J_{1',2'}$ 8.4 Hz, 1'-H), 5.00 (1 H, dd, $J_{3',4'}$ 2.8 and $J_{2',3'}$ 9.6 Hz, 3'-H), 5.21 (1 H, t, $J_{2',3'}$ 9.6 Hz, 2'-H), 5.37 (1 H, d, $J_{3',4'}$, 4'-H), 5.75 (1 H, br d, *J* 6.6 Hz, N*H*).

and (24) (170 mg, 35%); $\delta_H$ ($CDCl_3$) 0.6-1.5 (15 H, 3xm, octyl *H*), 1.95 (3 H, s, O*Ac*), 1.96 (3 H, s, O*Ac*), 1.99 (3 H, s, O*Ac*), 2.05 (3 H, s, O*Ac*), 2.06 (3 H, s, O*Ac*), 2.13 (3 H, s, NH*Ac*), 3.14 (1 H, m, -OC$H_2$ of octyl), 3.4-3.9 (8 H, m), 4.27 (2 H, m), 4.57 (2 H, m), 4.85 (1 H, t, $J_{3,4}$ 9.1 Hz, 4-H), 4.9-5.1 (3 H, m), 5.33 (1 H, d, $J_{3',4'}$ 3.0 Hz, 4'-H), 5.86 (1 H, br d, *J* 7.2 Hz, N*H*).

*Octyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-4-O-sulfonato-β-D glucopyranoside* (25).

Sulfation of alcohol (23) was carried out as described for sulfate (11) using alcohol (23) (76mg, 0.11 mmol), pyridine (5 $cm^3$) and sulfur trioxide-trimethylamine (56 mg, 0.41 mmol). Deprotection and purification of the residue obtained was carried out as described for sulfate (15). The *title compound* was obtained as a white solid (48 mg, 75%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.9 (3 H, s, NH*Ac*), 3.2-4.0 (12 H, m), 4.01 (1 H, dd, $J_{6a,b}$ 12.1 and $J_{5,6b}$ 2.3 Hz, 6b-*H*), 4.1 (1 H, t, $J_{3,4}$ 9.1 Hz, 3-*H*), 4.28 (1 H, t, $J_{3,4}$, 4-*H*), 4.45 (1 H, d, $J_{1,2}$ 8.05 Hz, 1-*H*), 4.58 (1 H, d, $J_{1',2'}$ 8.6 Hz, 1'-*H*); $\delta_C$ ($D_2O$), 13.4, 22.0, 22.3, 25.1, 28.3, 28.5 (2xC), 31.1, 55.4, 60.9, 61.1, 68.9, 70.7, 72.8, 74.8, 74.9, 75.1, 78.1, 100.9, 103.7, 104.2, 174.6 (C=O); FAB-MS: Calcd for [$C_{22}H_{41}NO_{14}S$.Na] 597.61: *m/z* 619.7 (M+Na).

*Octyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-4-O-phosphonato-β-D glucopyranoside* (26).

Alcohol (23) was phosphorylated as described for compound (12) using alcohol (23) (100 mg, 0.14 mmol), 1-*H* tetrazole (30 mg, 0.43 mmol), *N,N*-diisopropyldibenzyl phosphoramidite (0.07 $cm^3$, 0.21 mmol) and *m*CPBA (50 mg, 0.30 mmol). Deprotection and purification of the oil obtained was carried out as described for compound (16) yielding *phosphate (26)* as a white solid (62 mg, 70%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.9 (3 H, s, NH*Ac*), 3.4-3.8 (12 H, m), 4.04 (2 H, m, 4-*H*, 6a-*H*), 4.5 (1 H, d, $J_{1,2}$ 8.0 Hz, 1-*H*), 4.56 (1 H, d, $J_{1',2'}$ 8.6 Hz, 1'-*H*); $\delta_C$ ($D_2O$), 13.4, 22.0, 22.4, 25.1, 28.3, 28.5, 28.6, 31.1, 55.3,

60.6, 61.0, 68.6, 70.7 (2xC), 71.9, 72.5, 75.7, 80.6, 101.1, 104.2; $\delta_P$ ($D_2O$), 2.8; FAB-MS: Calcd for [$C_{22}H_{41}NO_{14}P.Na_2$] 619.5 obs: *m/z* 619.8 (M+1).

*Octyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-6-O-sulfonato-β-D glucopyranoside* (27).

Sulfation of alcohol (24) was carried out as described for sulfate (11) using alcohol (24) (130 mg, 0.18mmol), pyridine (10 $cm^3$) and sulfur trioxide-trimethylamine complex (75 mg, 0.53 mmol). Deprotection and purification were carried out as described for sulfate (15) yielding *sulfate (27)* as a white solid (96 mg, 88%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.9 (3 H, s, NH*Ac*), 3.3-3.8 (12 H, m), 4.23 (1 H, dd, $J_{6a,b}$ 11.0 and $J_{5,6a}$ 5.4 Hz, 6a-*H*), 4.38 (1 H, dd, $J_{6a,b}$ and $J_{5,6b}$ 1.74 Hz, 6b-*H*), 4.45 (1 H, d, $J_{1,2}$ 8.1 Hz, 1-*H*), 4.59 (1 H, d, $J_{1',2'}$ 8.1 Hz, 1'-*H*); $\delta_C$ ($D_2O$), 13.4, 22.0, 22.3, 25.0, 28.3, 28.5 (2xC), 31.1, 54.6, 61.1, 67.2, 68.6, 70.7 (2xC), 72.5, 73.3, 75.3, 82.2, 101.0, 103.6; ES-MS: Calcd for [$C_{22}H_{41}NO_{14}S$] 574.6 obs: *m/z* 574.0 (M-1).

*Octyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-6-O-phosphonato-β-D glucopyranoside* (28).

Alcohol (24) was phosphorylated as described for compound (12) using alcohol (23) (100 mg, 0.14 mmol), 1-*H* tetrazole (30 mg, 0.43 mmol), *N,N*-diisopropyldibenzyl phosphoramidite (0.07 $cm^3$, 0.21 mmol) and *m*CPBA (50 mg, 0.30 mmol). Deprotection and purification of the oil obtained was carried out as described for compound (16) yielding *phosphate* (26) as a white solid (88 mg, 99%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.90 (3 H, s, NH*Ac*), 3.3-3.95 (12 H, m), 4.01-4.11 (1 H, m, 6a-*H*), 4.19 (1 H, dd, $J_{6a,b}$ 11.1 and $J_{5,6b}$ 5.21 Hz, 6b-*H*), 4.46 (1 H, d, $J_{1,2}$ 8.1 Hz, 1-*H*), 4.58 (1 H, d, $J_{1',2'}$ 8.1 Hz, 1'-*H*); $\delta_C$ ($D_2O$), 13.4, 22.0, 22.2, 25.0, 28.3, 28.5, 31.1, 54.7, 61.1, 64.0, 68.4, 68.6, 70.7 (2xC), 72.6, 74.4 (2xC), 75.3, 82.0, 101.0; $\delta_P$ ($D_2O$), 5.3; ES-MS: Calcd for [$C_{22}H_{42}NO_{14}P$] 574.53 obs: *m/z* 572.9 (M-1).

*Octyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-4,6-di-O-sulfonato-β-D glucopyranoside* (**29**).

Sulfation of diol (**22**) was carried out as described for sulfate (**11**) using alcohol (**22**) (55 mg, 0.08 mmol), pyridine (3 $cm^3$) and sulfur trioxide-trimethylamine complex (90 mg, 0.66 mmol). Deprotection and purification were carried out as described for sulfate (**15**) yielding *di-O-sulfate (29)* as a white solid (49 mg, 88%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 2.05 (3 H, s, NH*Ac*), 3.54 (1 H, m, -OC$H_2$ octyl), 3.6-3.8 (5 H, m), 3.8-4.0 (5 H, m), 4.1-4.2 (2 H, m, 6a-*H*, 6b-*H*), 4.26 (1 H, t, $J_{3,4}$ 8.7 Hz, 4-*H*), 4.5 (1 H, d, $J_{1,2}$ 7.5 Hz, 1-*H*), 4.6 (1 H, d, $J_{1',2'}$ 8.1 Hz, 1'-*H*); $\delta_C$ ($D_2O$) 10.1, 18.7, 19.1, 21.8, 25.0, 25.2 (2xC), 27.8, 51.9, 57.8, 64.5, 65.7, 67.4, 67.5, 69.5, 69.6, 71.6, 71.9, 74.7, 97.5, 100.4, 171.3 (C=O); ES-MS: Calcd for [$C_{22}H_{41}NO_{17}S_2$] 654.66 obs: *m/z* 327.9 ($M^{2-}$).

*Octyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-4,6-di-O-phosphonato-β-D glucopyranoside* (**30**).

Diol (**22**) was phosphorylated as described for compound (**12**) using alcohol (**22**) (55 mg, 0.08 mmol), 1-*H* tetrazole (30 mg, 0.43 mmol), *N,N*-diisopropyldibenzyl phosphoramidite (0.08 $cm^3$, 0.24 mmol) and *m*CPBA (57 mg, 0.33 mmol). Deprotection and purification of the oil obtained was carried out as described for compound (**16**) yielding *phosphate* (**30**) as a white solid (58 mg, 99%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 2.04 (3 H, s, NH*Ac*), 3.5-3.8 (5 H, m), 3.8-4.0 (5 H, m), 4.06 (2 H, m, 6a-*H*, 6b-*H*), 4.16 (1 H, dd, $J_{4,5}$ and $J_{3,4}$ 9.3 Hz, 4-*H*), 4.51 (1 H, d, $J_{1,2}$ 7.0 Hz, 1-*H*), 4.59 (1 H, d, $J_{1',2'}$ 8.7 Hz, 1'-*H*); $\delta_C$ ($D_2O$) 10.1, 18.7, 19.1, 21.8, 25.1, 25.2 (2xC), 27.8, 51.8, 57.7, 60.6, 65.4, 67.3, 67.5, 68.7, 69.3, 71.2, 72.2, 76.7, 97.8, 100.2, 171.3 (C=O); $\delta_P$ ($D_2O$) 3.7, 6.1; ES-MS: Calcd for [$C_{22}H_{43}NO_{17}P_2$] 655.49 obs: *m/z* 327.6 ($M^{2-}$).

*Octyl 2-acetamido-2-deoxy-4-O-( β-D-galactopyranosyl )-β-D glucopyranoside (31).*

A solution of compound (**13**) (100 mg, 0.15 mmol) in MeOH (10 $cm^3$) containing NaOMe (10 mg) was stirred at r.t. for 4 h. Amberlite IR-120 ($H^+$) resin was added and the mixture was stirred until neutralisation had occurred. The suspension was filtered and the filtrate was concentrated to dryness to give the *title compound* as a crunchy solid (70 mg, 94%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 2.05 (3 H, s, NH*Ac*), 3.5-3.95 (10 H, m),

4.01 (1 H, br d, $J_{6a,b}$ 11.6 Hz, 6b-*H*); $\delta_C$ ($D_2O$), 13.2, 21.6, 25.7, 29.1, 29.2 (2xC), 31.6, 55.4, 60.6, 61.1, 69.0, 69.3, 71.3, 72.9, 73.5, 75.2, 75.8, 79.7, 101.5, 103.8; FAB-MS: Calcd for [$C_{22}H_{42}NO_{11}$] 495.55 obs: *m/z* 496 (M+1), 517.9 (M+Na).

*Octyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-β-D glucopyranoside (32).*

A solution of compound (22) (105 mg, 0.15 mmol) in MeOH (10 $cm^3$) containing NaOMe (10 mg) was stirred at r.t. for 4 h. Amberlite IR-120 ($H^+$) resin was added and the mixture was stirred until neutralisation had occurred. The suspension was filtered and the filtrate was concentrated to dryness to give the *title compound* as a crunchy solid (76 mg, 96%); $\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 2.04 (3 H, s, NH*Ac*), 3.4-3.7 (5 H, m), 3.7-3.85 (5 H, m), 3.92 (2 H, m, 6a-*H*, 6b-*H*), 4.44 (1 H, d, $J_{1,2}$ 7.6 Hz, 1-*H*), 4.57 (1 H, d, $J_{1',2'}$ 7.6 Hz, 1'-*H*); $\delta_C$ ($D_2O$), 11.5, 20.1, 20.4, 23.2, 26.4, 26.6, 29.2, 52.7, 58.8, 59.1, 66.6, 66.8, 68.7, 68.8, 70.6, 73.4, 73.5, 80.6, 99.0, 101.6, 172.6 (C=O); FAB-MS: Calcd for [$C_{22}H_{42}NO_{11}$] 495.55 obs: *m/z* 496 (M+1), 517.9 (M+Na).

*Octyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-methanesulfonyl-β-D-glucopyranoside* **(33)**

Compound (33) was prepared by sulfonylation of compound (8) using a procedure described by Vasella and co-workers [6]. To a solution of octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (8) (1.44 g, 3.42 mmol) and $Et_3N$ (0.72 $cm^3$, 5.13 mmol) in $CH_2Cl_2$ (10 $cm^3$) was added a solution of methanesulfonyl chloride (0.44 $cm^3$, 5.7 mmol) in $CH_2Cl_2$ (5 $cm^3$). Once t.l.c. (toluene-EtOAc, 4:1) showed the reaction to be complete (1 h) $CH_2Cl_2$ was added and the solution was washed with $H_2O$ (3x20 $cm^3$) dilute NaOH solution (20 $cm^3$) and $H_2O$ (20 $cm^3$). The organic layer was dried over $Na_2SO_4$ and concentrated to dryness. Crystallisation yielded white needles of the *title compound* (1.46 g, 85%); m.p. 172-174 °C ($CH_2Cl_2$ / hexane); $[\alpha]_D$ -35.8 (*c* 0.1, $CHCl_3$); (Found, C, 57.47; H, 7.53; N, 2.78. $C_{24}H_{37}O_8NS$ requires C, 57.69; H, 7.46; N, 2.80); $\delta_H$ ($CDCl_3$), 0.6-1.5 (15 H, 3xm, octyl *H*), 2.03 (3 H, s, NH*Ac*), 2.93 (3 H, s, -$SO_2$*Me*), 3.3-3.9 (6 H, m), 4.40 (1 H, dd, $J_{5,6'}$ 9.3 and $J_{6,6'}$ 10.4 Hz, 6'-*H*), 5.15 (1 H, d, $J_{1,2}$ 8.1 Hz, 1-*H*), 5.26 (1 H, dd, $J_{2,3}$ 9.3 and $J_{3,4}$ 9.5 Hz, 3-*H*), 5.54 (1 H, s, ArC*H*), 5.86 (1 H, d, *J* 7.7 Hz, N*H*), 7.52 (5 H, m, *Ar*).

*Octyl 2-acetamido-4,6-O-benzylidene-2-deoxy-β-D-allopyranoside* (34)

Displacement of the 3-*O*-methanesulfonyl group of (33) to give the *allo*-configured alcohol was carried out using a procedure used by Vasella and co-workers [6]. A solution of (33) (53 mg, 1.00 mmol), 2-methoxyethanol (6 $cm^3$), NaOAc (41 mg, 5.00 mmol) and $H_2O$ (0.3 $cm^3$) was refluxed at 115 ° C under argon. After 20 h t.l.c. (toluene/EtOAc, 2:1) showed the reaction to be complete. $CH_2Cl_2$ was added and the solution was washed with $H_2O$ (2x15 $cm^3$), dried over $Na_2SO_4$, and concentrated to dryness until almost all the $CH_2Cl_2$ had evaporated. Hexane was added and white needles of the *title compound* formed (45 mg, 98%); m.p. 186-188 ° C; $[\alpha]_D$ -65 (*c* 0.1, $CHCl_3$); (Found C, 65.59; H, 8.45; N, 3.29. $C_{23}H_{35}O_6N$ requires C, 65.53; H, 8.37; N, 3.33); $\delta_H$ ($CDCl_3$), 0.6-1.5 (15 H, 3xm, octyl *H*), 2.02 (3 H, s, NH*Ac*), 3.42 (1 H, m, -OC$H_2$ octyl chain ), 3.6-4.0 (4 H, m), 4.14 (1 H, m, 5-*H*), 4.25 (1-H, br s, 3-*H*), 4.35 (1 H, dd, $J_{3,4}$ 3.0 and $J_{4,5}$ 6.0 Hz, 4-*H*), 4.63 (1 H, d, $J_{1,2}$ 8.4 Hz, 1-*H*), 5.59 (1 H, s, ArC*H*), 5.96 (1 H, br d, *J* 8.6 Hz, N*H*), 7.2-7.4 (5 H, m, *Ar*).

*Octyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-methanesulfonyl-β-D-allopyranoside* (35)

Compound (35) was prepared using the procedure described for compound (33) above. To a solution of octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-allopyranoside (34) (100 mg, 0.24 mmol) and $Et_3N$ (0.05 $cm^3$, 0.35 mmol) in $CH_2Cl_2$ (5 $cm^3$) was added a solution of methanesulfonyl chloride (0.03 $cm^3$, 0.35 mmol) in $CH_2Cl_2$ (2 $cm^3$). Once t.l.c. (toluene/EtOAc, 4:1) showed the reaction to be complete (10 min), $CH_2Cl_2$ was added and the solution was washed with $H_2O$ (3x20 $cm^3$), dilute NaOH solution (20 $cm^3$) and $H_2O$ (20 $cm^3$). The organic layer was dried over $Na_2SO_4$ and concentrated to dryness. Crystallisation yielded white needles of the *title compound* (85 mg, 72%); m.p. 166-168 ° C ($CH_2Cl_2$ / hexane); $[\alpha]_D$ -29.6 (*c* 0.05, $CHCl_3$); (Found, C, 57.56; H, 7.45; N, 2.86. $C_{24}H_{37}O_8S$ requires C, 57.69; H, 7.46; N, 2.80); $\delta_H$ ($CDCl_3$), 0.6-1.5 (15 H, 3xm, octyl *H*), 2.04 (3 H, s, NH*Ac*), 2.98 (3 H, s, -$SO_2$*Me*), 3.46 (1 H, dt, *J* 2.6 and 6.7 Hz, -OC$H_2$ octyl chain), 3.74-3.94 (3 H, m), 4.28 (1 H, dt, $J_{2,3}$ 2.8 and $J_{1,2}$ 8.7 Hz, 2-*H*), 4.41 (1 H, dd, $J_{5,6'}$ 4.2 and $J_{6,6'}$ 9.6 Hz, 6'-*H*), 4.67 (1 H, d, $J_{1,2}$, 1-*H*), 5.27 (1 H, dd, $J_{2,3}$ and $J_{3,4}$ 2.6 Hz, 3-*H*), 5.58 (1 H, s, ArC*H*), 5.73 (1 H, d, *J* 8.4 Hz, N*H*), 7.36 (5 H, m, Ar*H*).

*Octyl 2-acetamido-3-azido-4,6-O-benzylidene-2,3-di-deoxy-β-D-glucopyranoside* (36)

Compound (36) was prepared using a procedure described by Wong and co-workers [7]. A suspension of octyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulfonyl-β-D-allopyranoside (35) (80 mg, 0.2 mmol) and $NaN_3$ (130 mg, 2 mmol) in DMF (2 $cm^3$) was stirred at 110 °C for 4 h. The solution was cooled to r.t. and $CHCl_3$ was added. The mixture was washed with $H_2O$ (2x10 $cm^3$) and NaCl (10 $cm^3$). The organic layer was dried over $Na_2SO_4$, concentrated to dryness, and separated by flash chromatography (toluene/EtOAc, 1:1). Crystallisation gave white needles of the *title compound* (60 mg, 65%); m.p. 226-229 °C (EtOH); $[\alpha]_D$ 23.0 (*c* 0.47, $CHCl_3$); (Found C, 61.99; H, 7.83; N, 12.25. $C_{23}H_{35}O_5N_4$ requires C, 61.86; H, 7.67; N, 12.55); $\nu_{max}$ 3260 (NH), 2105 ($N_3$), 1560 (CO); $\delta_H$ ($CDCl_3$) 0.6-1.5 (15 H, 3xm, octyl *H*), 2.04 (3 H, s, NH*Ac*), 3.20 (1 H, m, -OC*H*$_2$ octyl chain), 3.4-3.6 (3 H, m), 3.7-3.9 (2 H, m), 4.36 (1 H, dd, $J_{5,6'}$ 4.3 and $J_{6,6'}$ 10.3 Hz, 6'-*H*), 4.50 (1 H, t, $J_{2,3}$ and $J_{3,4}$ 10.5 Hz, 3-*H*), 5.04 (1 H, d, $J_{1,2}$ 8.1 Hz, 1-*H*), 5.57 (1 H, s, ArC*H*), 7.3-7.4 (5 H, m, Ar*H*).

*Octyl 2-acetamido-3-azido-6-O-benzyl-2,3-di-deoxy-β-D-glucopyranoside* (37)

Compound (37) was prepared using a selective acetal ring opening procedure described by DeNinno and co-workers [5]. To an ice-cold suspension of octyl 2-acetamido-3-*O*-azido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (36) (50 mg, 0.11mmol) and triethylsilane (0.09 $cm^3$, 0.55 mmol) in $CH_2Cl_2$ (2 $cm^3$), TFA (0.04 $cm^3$, 0.55 mmol) was added dropwise. The solution was allowed to warm to r.t. Once t.l.c. (toluene/EtOAc, 1:1) showed the reaction to be complete (4 h), EtOAc was added and the solution was subjected to a standard work up. The product was purified by flash chromatography (toluene/EtOAc, 2:1) to afford the *alcohol* (37) as white needles (47 mg, 93%); m.p. 110-112 °C; $[\alpha]_D$ -16 (*c* 0.47, $CHCl_3$); (Found C, 61.65; H, 8.38; N, 12.62. $C_{23}H_{36}O_5N$ requires C, 61.59; H, 8.09; N, 12.49.); $\nu_{max}$ 3265 (NH), 2110 (N3), 1655 (CO); $\delta_H$ ($CDCl_3$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.94 (3 H, s, NH*Ac*), 2.98 (1 H, m, -OC*H*$_2$ octyl chain), 3.30-3.55 (3 H, m), 3.60-3.80 (3 H, m), 4.13 (1 H, dd, $J_{2,3}$ and $J_{3,4}$ 9.7 Hz, 3-*H*), 4.51 (2 H, dd, 12.0 Hz, ArC*H*$_2$), 4.86 (1 H, d, $J_{1,2}$ 8.0 Hz, 1-*H*), 5.64 (1 H, d, *J* 6.6 Hz, N*H*), 7.24 (5 H, m, Ar*H*).

## *4.2 References*


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# *Chapter 5: Results and Discussion (Biological)*

## *5.1 Biotransformations*

Since the discovery that carbohydrates are involved in many processes in biological systems, there has been an increased interest in the preparation of naturally occurring oligosaccharides. New techniques and methods of analysis have simplified the task of the organic chemist in synthesis of such complex oligosaccharides. However, chemical synthesis of oligosaccharides involves many protecting group manipulations, and coupling reactions are typically low yielding. To make a single monosaccharide building block takes an average of five synthetic steps (at approximately one step per week). Therefore the task of preparing complex oligosaccharides synthetically is both time consuming and expensive. The chemo-enzymatic synthesis of carbohydrate molecules has therefore many advantages. The specificity and catalytic power of enzymes can be exploited to carry out regio- and stereo-controlled coupling reactions on unprotected or partially protected compounds. This reduces the number of chemical steps necessary in the synthesis of oligosaccharides. The disadvantage is in the range of substrates specific enzymes can tolerate and in some cases (for example, the synthesis of sulfate and phosphate derivatives of octyl LacNAc) a chemical method is the only suitable method to synthesise unnatural molecules.

There are several different methods for creating glycosidic linkages enzymatically. The enzymes typically used are glycosidases, phosphorylases and glycosyltransferases [1]. The donor molecules frequently employed for glycosidases are free sugars, glycosides, phenyl glycoside derivatives and glycosyl fluorides. Phosphorylases use sugar-1-phosphates as donors and glycosyltransferases require the naturally occurring sugar nucleotide donors. Studies have been carried out to determine the acceptor specificities for many glycosyl transferase enzymes and it has been found that for some enzymes there are *key* hydroxyl groups involved in recognition and binding to the enzyme [2] - [3]. Only these *key* polar groups cannot be altered without adversely effecting the binding and rate of reaction. Substitutions at other sites of the acceptor sugar can be tolerated, enabling the enzymatic synthesis of unnatural oligosaccharides.

### 5.1.1 Chemo-Enzymatic Synthesis of Octyl LacNAc

The enzymatic synthesis of octyl LacNAc uses octyl GlcNAc (7) as an acceptor which was prepared chemically in three steps as discussed in **Chapter 3**. The method chosen for the enzymatic glycosidation using bovine β-1,4-GalT (EC 2.4.1.22) was a variation of a method described by Wong and co-workers [4], [5] and Flitsch and co-workers [6]. The expensive donor in the reaction, UDP-Gal, was generated *in situ* using UDP-Glc and UDP-Glc-4-epimerase. A problem encountered was the poor solubility of the acceptor substrate in the assay buffer. Despite sonication, heating and addition of 10% v/v DMSO, the acceptor remained a gel in the assay mixture. However, galactosylation of octyl GlcNAc was achieved in 56% yield in 24 h. Analytical data was identical to that obtained for synthetically prepared (31).

Synthesis of octyl LacNAc using a chemo-enzymatic method was overall less time consuming, less expensive and higher yielding than the corresponding chemical method involving eleven steps (overall yield from octyl GlcNAc 22%).

### 5.1.2 Synthesis of α-2,3-Sialyl Octyl LacNAc Analogues

The synthesis of the sialylated derivatives **36-39** (**Figure 5-1**) was carried out using a glycosidase enzyme, *trans*-sialidase (from *Trypanosoma cruzi*) [7]. The donor used in the reaction was the *p*-nitrophenyl glycoside of sialic acid (PNP-NeuAc ) which was available in our laboratories.

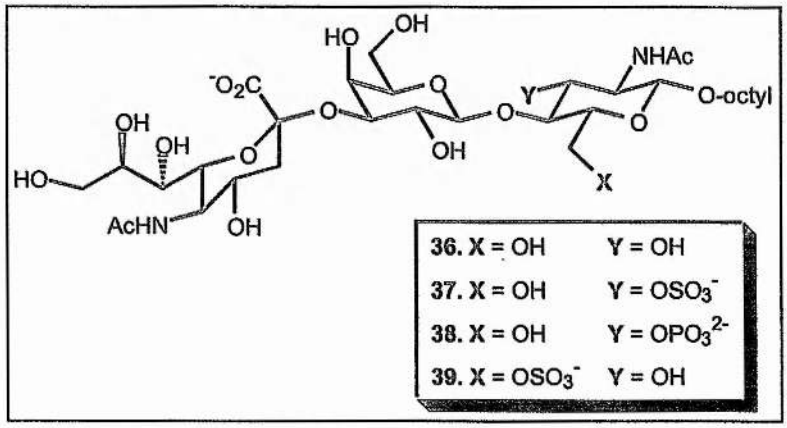


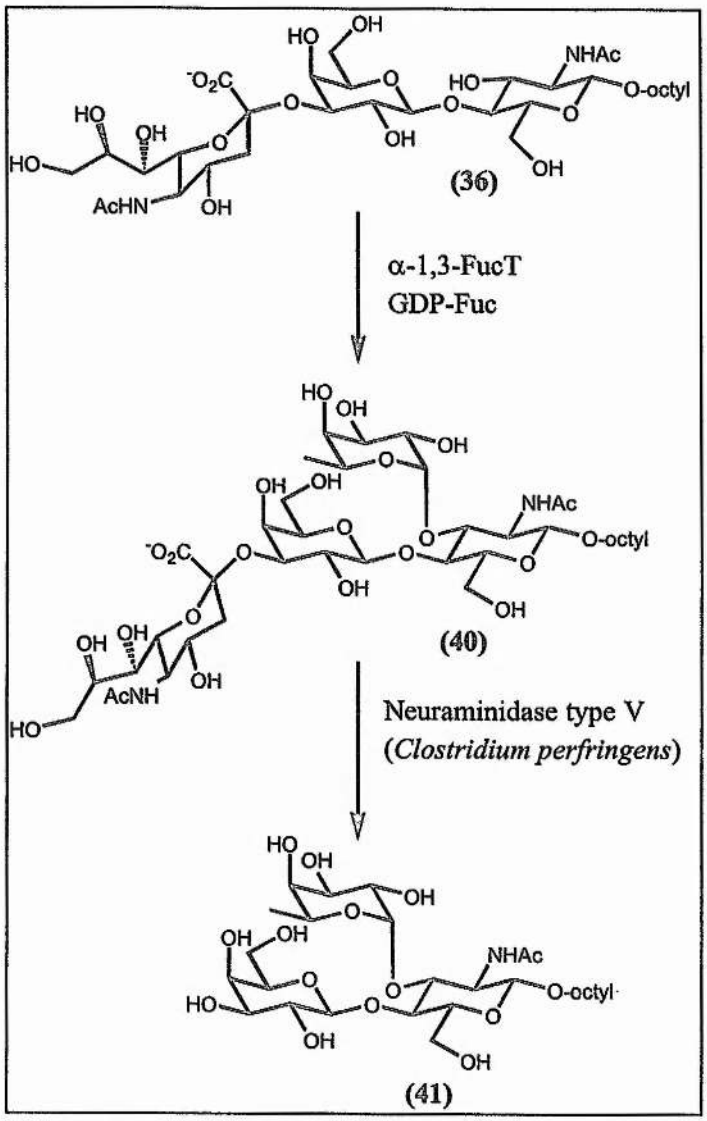

**Figure 5-1: Sialylated Octyl LacNAc Substrates**

Attempts were also made to sialylate the 6-*O*-phosphate (**19**), 3,6-di-*O*-sulfate (**18**) and 3,6-di-*O*-phosphate (**20**) derivatives of octyl LacNAc but no turnover was observed by t.l.c. This was thought to be due to the highly charged nature of the substrates, perhaps causing tight solvation of the molecules and interfering with binding to the enzyme. Experiments are currently being carried out to investigate this possibility.

Purification of the compounds (**36**)-(**39**) (**Figure 5-1**) was carried out by passage through QAE-Sephadex AG-25 as described in **Chapter 3**. However, a side product from the assay was *p*-nitrophenol which eluted with the sialylated compounds from the SepPak cartridges. Removal of *p*-nitrophenol was achieved by column chromatography using a size exclusion lipophilic Sephadex LH-20 resin using methanol as the mobile phase.

### 5.1.3 Chemo-Enzymatic Synthesis of Octyl Lewis x

Octyl Lewis x (**41**) was prepared according to **Scheme 5-1**. This order of events was chosen over direct fucosylation of octyl LacNAc due to the higher turnover observed when sialyl octyl LacNAc was used as a substrate with α-1,3-FucT. The synthesis makes use of readily available human milk Lewis α-1,3-FucT (kindly provided by M. M. Palcic) and commercially available neuraminidase type V (from *Clostridium perfringens*).




**Scheme 5-1: Synthesis of Octyl Lewis x**

## *5.2 Enzyme Kinetics*

To gain information regarding the substrate specificity of α-1,3-FucTs the compounds shown in **Figure 5-1, Figure 5-2** and **Figure 5-3** were to be used as substrates to screen for activity with semi-pure α-1,3/4-FucT (human milk) and five recombinant α-1,3-FucT's (III-VII, gratefully received as a gift from Dr. C. Britten, GlaxoWellcome).

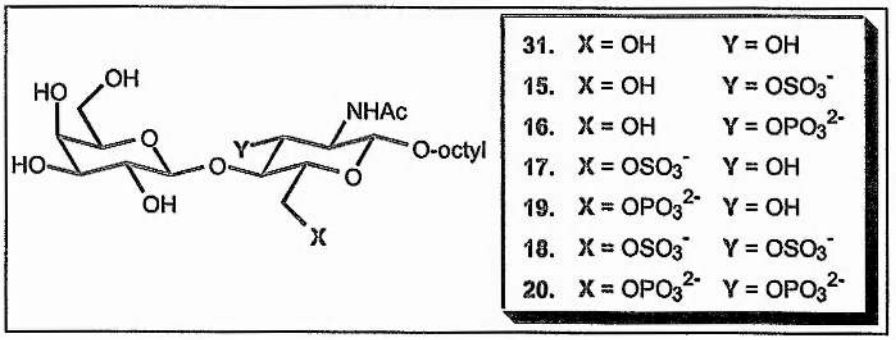



**Figure 5-2: Octyl LacNAc Substrates**

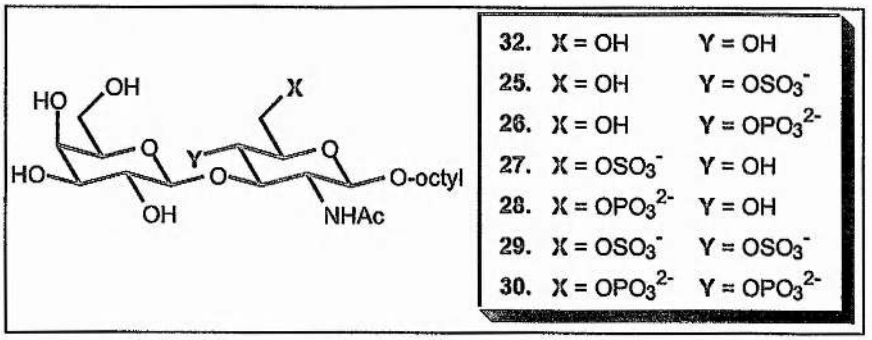



**Figure 5-3: Octyl Gal-β-1,3-GlcNAc Substrates**

These compounds all contain an octyl chain which allows the product from the enzymatic reaction to be separated from starting materials and enzyme by reverse phase chromatography using $C_{18}$ SepPak cartridges [8]. In order to detect product formation, a radiochemical assay system described by Palcic and co-workers [8] was chosen. The radiochemical donor used was GDP-[$^3$H]-fucose. The kinetic experiments were carried out at 37°C, quenched with water and loaded onto a SepPak cartridges. Only compounds containing a hydrophobic residue adhered to the cartridges. All other substances present in the assay mixture were washed from the SepPak with water. Any radiolabelled product

formed during the assay was then eluted from the cartridge with MeOH, diluted with scintillation cocktail and counted in a scintillation counter. The Michaelis-Menten equation was then applied to determine $K_M$ and $V_{max}$ values using a Lineweaver-Burk plot. However Michaelis-Menten kinetics applies to reactions involving a ***single*** substrate. For α-1,3-FucT reactions two substrates are required, the acceptor substrate and the donor, GDP-Fuc. To overcome this problem, donor concentrations were used at saturation level and can thus be regarded as constant.

The studies reported in this chapter make use of a number of recombinant fucosyltransferases. Due to the poor availability of the corresponding native enzymes it is not possible to do detailed kinetic comparisons between two forms of the same enzyme. It is therefore inappropriate to quantitatively compare turnover data ($V_{max}$) for different substrates with different recombinant enzymes and draw 'physiologically relevant' conclusions. The data reported are therefore interpreted in a conservative fashion, with relative Vmax/K values quoted rather than $k_{cat}$ values.

**Table 5-1** to **Table 5-6** give the results obtained when the compounds shown in **Figure 5-1** to **Figure 5-3** were screened against all of the α-1,3-FucT's.

### 5.2.1 Lewis α-1,3/4-FucT (human milk)

| Substrate | $K_M$ (μM) | $V_{max}$ (pmol/min/μl) | Rel. $V_{max}/K_M$ |
|---|---|---|---|
| **Type I** | | | |
| Gal-β-1,3-GlcNAc-*O*R (32)* | 2560 | 30.0 | 85 |
| 4-*O*-sulfate (25) | 16900 | 1.15 | 0.5 |
| 4-*O*-phosphate (26) | 18800 | 0.53 | 0.2 |
| **Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (31) | 350 | 4.92 | 100 |
| 3-*O*-sulfate (15) | 3120 | 5.07 | 11 |
| 3-*O*-phosphate (16) | 2470 | 0.30 | 0.9 |

**Table 5-1: Kinetic Data for Lewis α-1,3/4-FucT**

The 6-*O*-sulfate and 6-*O*-phosphate compounds were not tested as substrates for this enzyme.

* R = $-(CH_2)_7CH_3$ (octyl) in this and all subsequent tables.

### 5.2.2 α-1,3-FucT III

| Substrate | $K_M$ (μM) | $V_{max}$ (pmol/min/mg) | Rel. $V_{max}/K_M$ |
|---|---|---|---|
| **Type I** | | | |
| Gal-β-1,3-GlcNAc-*O*R (32) | 1700 | 42640 | 100 |
| 6-*O*-sulfate (27) | 600 | 6980 | 46 |
| 6-*O*-phosphate (28) | 300 | 10850 | 144 |
| **Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (31) | >7000 | - | - |
| 6-*O*-sulfate (17) | 1500 | 780 | 2 |
| 6-*O*-phosphate (19) | 500 | 1320 | 11 |
| **Sialylated Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (36) | >1000 | - | - |
| 3-*O*-sulfate (37) | >1000 | - | - |
| 6-*O*-sulfate (39) | 7600 | 930 | 1 |
| 3-*O*-phosphate (38) | >1000 | - | - |

**Table 5-2: Kinetic Data for α-1,3-FucT III**

Accurate kinetic data were not obtained for the substrate Gal-β-1,4-GlcNAc-*O*R due to problems of insolubility. The maximum solubility of this substrate is 2mM which is much lower than the $K_M$ value. $K_M$ values were not determined for substrates with $K_M$ >10mM.

### 5.2.3 α-1,3-FucT IV

| Substrate | $K_M$ (μM) | $V_{max}$ (pmol/min/mg) | Rel. $V_{max}/K_M$ |
|---|---|---|---|
| **Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (31) | 1400 | 39 | 100 |
| 6-*O*-sulfate (17) | 1400 | 79 | 202 |
| 6-*O*-phosphate (19) | 500 | 16 | 115 |

**Table 5-3: Kinetic Data for α-1,3-FucT IV**

Compounds with substitutions at the 3-position of octyl LacNAc and sialylated derivatives were found to be non-substrates for α-1,3-FucT IV. Type I compounds were not tested as substrates for this enzyme.

### 5.2.4 α-1,3-FucT V

| Substrate | $K_M$ (μM) | $V_{max}$ (pmol/min/mg) | Rel. $V_{max}/K_M$ |
|---|---|---|---|
| **Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (31) | >5000 | - | - |
| 6-*O*-sulfate (17) | 9000 | 840 | 84 |
| 6-*O*-phosphate (19) | 2300 | 95 | 37 |
| **Sialylated Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (36) | 4400 | 490 | 100 |
| 3-*O*-sulfate (37) | 1300 | 10 | 7 |
| 6-*O*-sulfate (39) | 3000 | 360 | 108 |

**Table 5-4: Kinetic Data for α-1,3-FucT V**

Compounds with substitutions at the 3-position of octyl LacNAc were found to be non-substrates for α-1,3-FucT V. An exception was the sialylated 3-*O*-sulfate compound which had a $K_M$ smaller than that of the parent compound, sialylated octyl LacNAc. The

kinetic parameters for sialylated octyl LacNAc containing a phosphate at the 3-position could not be determined. When assays were carried out, a scatter of counts were observed with no correlation between duplicate experiments. A reason for this could be due to the large number of charges on the molecule. This would affect the affinity of the compound with the $C_{18}$ SepPak cartridges. In an attempt to overcome this problem, the SepPaks were treated with acid (distilled water acidified with TFA to a pH of 1.4) prior to the compound being loaded on. This method proved successful with highly charged substrates with α-1,3-FucT VI (**5.2.5**). However, for α-1,3-FucT V, no accurate data was obtained. Type I compounds were not tested as substrates for this enzyme.

### 5.2.5 α-1,3-FucT VI

| Substrate | $K_M$ (μM) | $V_{max}$ (pmol/min/mg) | Rel. $V_{max}$ /$K_M$ |
|---|---|---|---|
| **Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (31) | 115 | 2510 | 100 |
| 3-*O*-sulfate (15) | 495 | 5015 | 46 |
| 3-*O*-phosphate (16) | 785 | 1180 | 7 |
| 6-*O*-sulfate (17) | 0.85 | 3940 | 21260 |
| 6-*O*-phosphate (19) | 4.95 | 2420 | 2245 |
| 3,6-di-*O*-sulfate (18) | 390 | 945 | 11 |
| 3,6-di-*O*-phosphate (20) | 545 | 910 | 8 |
| **Sialylated Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (36) | 10 | 1205 | 555 |
| 3-*O*-sulfate (37) | 160 | 1035 | 30 |
| 3-*O*-phosphate (38) | 420 | 535 | 6 |
| 6-*O*-sulfate (39) | 4.4 | 1100 | 1150 |

**Table 5-5: Kinetic Data for α-1,3-FucT VI**

All of the compounds assayed with α-1,3-FucT VI proved to be good substrates for the enzyme. In particular compounds with substitutions at the 6-position proved to be

excellent substrates with $K_M$ values much lower (x1000) than the parent compound. The compounds with a sulfate or phosphate group at both the 3 and 6-positions have kinetic parameters similar to those of the 3-*O*-sulfate and phosphate derivatives. For the sialylated compounds, the 6-*O*-sulfate was also a good substrate, with kinetic parameters similar to those of the sialyl octyl LacNAc. Type I compounds were not tested as substrates for this enzyme.

### 5.2.6 α-1,3-FucT VII

| Substrate | $K_M$ (μM) | $V_{max}$ (pmol/min/mg) | Rel. $V_{max}$ /$K_M$ |
|---|---|---|---|
| **Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (31) | 110 | 19.9 | 100 |
| **Sialylated Type II** | | | |
| Gal-β-1,4-GlcNAc-*O*R (36) | 1320 | 33.1 | 14 |
| 3-*O*-sulfate (37) | 820 | 86.1 | 58 |
| 6-*O*-sulfate (39) | 280 | 450.3 | 888 |

**Table 5-6: Kinetic Data for α-1,3-FucT VII**

An interesting point was the observation that octyl LacNAc was an apparent substrate for α-1,3-FucT VII. This is contrary to previous reports [9], which suggested that α-1,3-FucT VII would only tolerate substrates containing an α-2,3-sialic acid moiety. Assays were carried out for the octyl LacNAc compounds containing sulfate and phosphate substitutions. Sulfate and phosphate substitutions at the 3-position rendered the compound inactive with α-1,3-FucT VII. Sulfate and phosphate substitutions at the 6-position appeared to be substrates for the enzyme (comparing directly all the compounds with α-1,3-FucT VII at 1mM concentrations, for 0.5 h incubations), however on carrying out $K_M$ determining experiments, no coherent results were obtained and no consensus between duplicate experiments was observed. This result can not yet be explained. Type I compounds were not evaluated as substrates for this enzyme.

### 5.2.7 Conclusion

It can be seen from the results shown in **Table 5-1 - Table 5-6** that the presence of an anionic functionality in the substrate molecules profoundly affects the $K_M$ and $V_{max}$ values for any given enzyme. In particular the presence of a sulfate group at position 6 of a substrate generally improves the $K_M$ value relative to the parent compound. An interesting observation is that for α-1,3-FucT VI the 3-*O*-sulfate (**15**) and 3-*O*-phosphate (**16**) derivatives are good substrates for the enzyme with $K_M$ and $V_{max}$ values in a similar range to those of the parent compound. This is in agreement with the results obtained by Palcic and co-workers (unpublished results) for the human milk α-1,3/4-FucT using LacNAc-MCO[†] 3-*O*-sulfate as a substrate. This is unexpected as the sulfate or phosphate moieties are at the site of glycosylation. An explanation could be that the fucose is transferring to the oxygen of the sulfate or phosphate groups to form sulfate and phosphate diester linkages as shown in **Figure 5-4**.

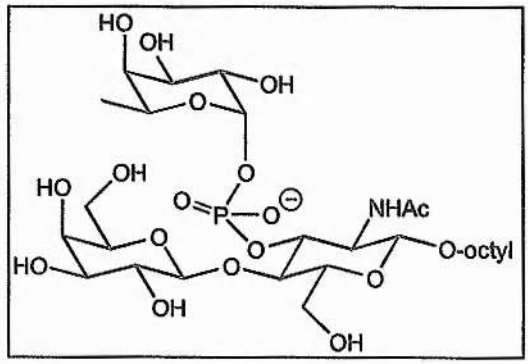



**Figure 5-4: Phosphate Diester Linkage**

#### *5.2.7.1 Verification of Phosphate Diester Linkage*

A sulfate diester linkage would be expected to be unstable, however, glycosyl phosphate diester linkages are known in nature and so should be stable to isolation [10]. Therefore studies were concentrated on identifying the product of the reaction of octyl LacNAc 3-*O*-phosphate (**16**) with α-1,3-fucosyltransferase. The enzyme chosen to study this was the Lewis α-1,3/4-FucT (human milk) as it was a highly concentrated enzyme and

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[†] MCO= 8-methoxycarbonyloct-1-yl

has previously been shown to be useful in large scale biotransformation reactions (5.1). To monitor the reaction MALDI-TOF was chosen. Using this technique, the enzymatic mixture could be directly measured without prior purification steps.

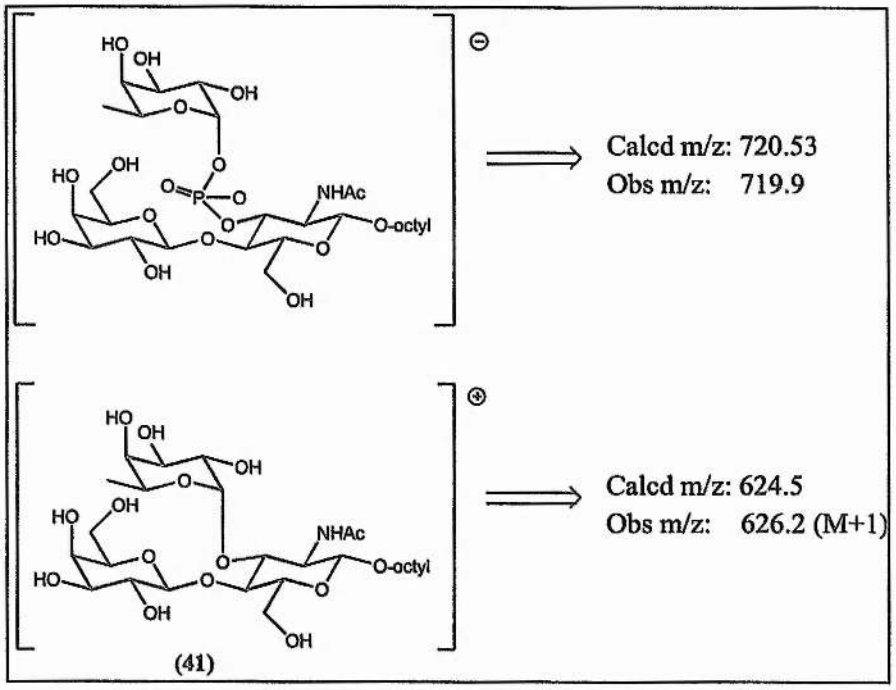


**Figure 5-5: Results of Attempted Characterisation of the Phosphate Diester**

The preliminary results of this assay did show the presence of a species of molecular weight corresponding to a fucosylated, phosphorylated octyl LacNAc derivative (**Figure 5-5**). It was also observed that there was a compound present with a molecular ion of 626.2. This corresponds to the trisaccharide octyl Lewis x (41). If the phosphate monoester was being cleaved octyl LacNAc would be formed which would be fucosylated to give octyl Lewis x. It was thought that the alkaline phosphatase present in the assay to degrade the GDP side product, could be responsible for the cleavage of the phosphate linkage. A second assay was set up containing no alkaline phosphatase. MALDI-TOF showed again the presence of a species which corresponded to a fucosylated, phosphorylated octyl LacNAc derivative. However, despite numerous attempts it was not possible to obtain sufficient material to isolate and characterize. A reason for this could be due to the build up of GDP which is an inhibitor of the enzyme.

A second strategy was adopted to verify the presence of the phosphate diester product. A radiochemical assay was set up using α-1,3-FucT VI and octyl LacNAc 6-*O*-phosphate (**19**). The product from this assay was loaded onto a t.l.c. plate. The t.l.c. was developed ($CHCl_3$/MeOH/$H_2O$, 10:10:3) and the silica gel was removed from the plate in 1 cm strips. Methanol was added and the suspensions were vortexed thoroughly, centrifuged and the supernatant removed. This process was repeated twice and the combined supernatants were counted. **Figure 5-6** shows the results obtained.

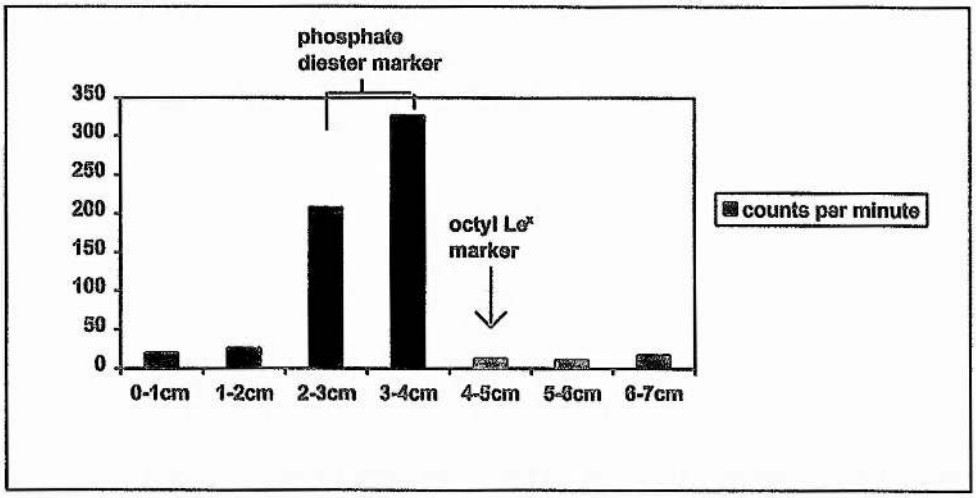


**Figure 5-6: Chart Showing the Silica Gel Bands Containing Radioactive Counts**

The sections 2-3 cm and 3-4 cm contained the highest number of radioactive counts (~ quantitative recovery of total number of counts loaded onto t.l.c. plate). This corresponded exactly with the $R_f$ of the authentic phosphate diester product (synthesised from phosphate (**16**) by Taketo Uchiyama in the laboratories of Prof. O. Hindsgaul, University of Alberta). The section corresponding to that of octyl Lewis x (section 4-5cm) contained only background levels of radioactivity. These results lend support to the notion that the phosphate group is being glycosylated in the fucosylation reaction with α-1,3/4-FucT VI.

### 5.2.8 Bovine β-1,4-GalT Kinetics

The compounds shown in **Figure 5-7** were synthesised as a model study to investigate the most suitable method for carrying out sulfation and phosphorylation reactions. GlcNAc derivatives are substrates for (bovine) D-glucose β-1,4-GalT (EC

2.4.1.22) and so the sulfate and phosphate derivatives of octyl GlcNAc were evaluated as substrates for β-1,4-GalT. The $K_M$ was found to be ~ 150μM for octyl GlcNAc. Accurate kinetic data was not obatined for this substrate due to problems of insolubility. Compounds containing a sulfate or phosphate moiety were non-substrates for the enzyme. This data agrees with previously reported results that substitutions at the 4-OH of GlcNAc are not tolerated by β-1,4-GalT [2].

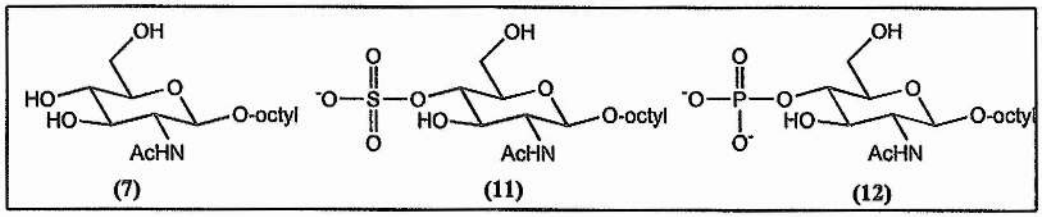


**Figure 5-7: Octyl GlcNAc Substrates for β-1,4-GalT (bovine)**

## *5.3 Experimental*

α-1,3/4-FucT used was semi-pure enzyme isolated from human milk by Prof. M. Palcic and co-workers at the University of Alberta. Recombinant α-1,3-FucT's were a gift from Dr. C. Britten at GlaxoWellcome. α-1,3-FucT III, IV and V were expressed by transient transfection in COS-1 cells. α-1,3-FucT VI and VII were expressed by infection of *Trichoplusia ni* by recombinant baculovirus. *Trans*-sialidase used was recombinant enzyme expressed in *E. Coli* by Jennifer Harrison in our laboratories. A standard preparation of this enzyme involved cells from a 10 L culture sonicated in 500 $cm^3$ of phosphate buffer, pH 8 containing 300 mM NaCl. The mixture was spun down and the supernatant aliquoted into 500 μl batches and frozen at -80 ° C.

*Enzymatic Synthesis of Octyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-β-D-glucopyranoside (31)*

A suspension of octyl 2-acetamido-2-deoxy-β-D-glucopyranoside (7) (60 mg, 0.18mmol) in HEPES buffer (6 $cm^3$, 20 mM HEPES, 20 mM $MnCl_2$ containing 0.2% bovine serum albumin) was sonicated for 15 mins. Alkaline phosphatase (40 μl, 10 U), UDP-glucose-4-epimerase (200 μl, 1 U), bovine β-1,4-GalT (EC 2.4.1.22) (200 μl, 2 U) and UDP-glucose (120 mg, 0.36 mmol) were added and the gel was incubated at 37 ° C until t.l.c. ($CHCl_3$/MeOH/$H_2O$, 10:10:3) indicated the no change in the reaction (24 h). Water was added and the mixture was purified by reverse phase chromatography on $C_{18}$ SepPak cartridges. Excess octyl GlcNAc (7) was removed by passage through lipophilic Sephadex LH20 using MeOH as an eluent. Concentration yielded a white solid (50mg, 56%) with analytical data identical to that obtained previously.

*General Procedure for Sialylation Reactions*

Octyl LacNAc derivatives (9 μmol) were dissolved in TRIS buffer (600 μl, 50 mM Trizma base at pH 7 containing 0.02% $NaN_3$) and PNP-NeuAc (18 μmol) and transsialidase (50 μl of a standard preparation) were added. The assay was incubated at 25 ° C until t.l.c. ($CHCl_3$/MeOH/$H_2O$, 10:10:3) indicated the reaction to be complete (96 h). The assay was diluted with $H_2O$ (1 $cm^3$) and loaded onto a QAE-Sephadex (anionic) column, eluted with

NaCl (1 M) directly onto a $C_{18}$ SepPak cartridge, washed with water and eluted with MeOH. The MeOH eluent was loaded onto a lipophilic Sephadex LH20 column and the fractions containing the sialylated compound were concentrated to dryness yielding a white solid (4-6 μmol, 44-71%).

The following compounds were prepared using the procedure outlined above:

*Octyl 2-acetamido-2-deoxy-4-O-[-(α-2,3'-5-acetamido-3,5-dideoxy-D-glycero-α-galacto-non-2-ulopyranosyl)-β-D-galactopyranosyl]-β-D-glucopyranoside (36)*

$\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.8 (1 H, t, $J_{3"a,e}$ 4.3 and $J_{3"a,4"}$ 12.2Hz, 3"-*H*a), 2.05 (6 H, s, NH*Ac*), 2.80 (1 H, dd, $J_{3"a,e}$ and $J_{3"e,4"}$ 4.2Hz, 3"-*H*e), 3.5-3.95 (28 H, m), 4.01 (1 H, dd, $J_{6a,b}$ 11.6 and $J_{5,6b}$ <2Hz, 6b-*H*), 4.38 (1 H, d, $J_{1,2}$ 7.8Hz, 1-*H*), 4.44 (1 H, d, $J_{1',2'}$ 7.9Hz, 1'-*H*); ES-MS: Calcd for [$C_{33}H_{58}O_{19}N_2$] 786.0 obs: *m/z* 785.7 (M-1).

*Octyl 2-acetamido-2-deoxy-4-O-[-(α-2,3'-5-acetamido-3,5-dideoxy-D-glycero-α-galacto-non-2-ulopyranosyl)-β-D-galactopyranosyl]-3-O-sulfonato-β-D-glucopyranoside (37)*

$\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.8 (1 H, t, $J_{3"a,e}$ 4.6 and $J_{3"a,4"}$12.0Hz, 3"-*H*a), 2.01 (3 H, s, NH*Ac*), 2.05 (3 H, s, NH*Ac*), 2.79 (1 H, dd, $J_{3"a,e}$ and $J_{3"e,4"}$ 4.6Hz, 3"-*H*e), 3.5-3.8 (13 H, m), 3.8-4.0 (13 H, m), 4.1 (1 H, dd, $J_{6a,b}$ 12.0 and $J_{5,6b}$ 4.6Hz, 6b-*H*), 4.5 (1 H, t, $J_{3,4}$ and $J_{2,3}$ 10.4Hz, 3-*H*), 4.52 (1 H, d, $J_{1,2}$ 7.5Hz, 1-*H*), 4.64 (1 H, d, $J_{1',2'}$ 8.1Hz, 1'-*H*); $\delta_C$ ($D_2O$), 11.5, 20.1, 20.4, 23.1, 26.4, 26.6 (2xC), 29.2, 37.7, 49.7, 52.7, 58.0, 59.o, 60.6, 65.8, 66.2, 66.4, 67.4, 68.7, 69.8, 71.0, 73.0, 73.1, 74.0, 74.3, 77.4, 97.8, 98.5, 101.3, 102.5, 171.8 (C=O), 172.4 (C=O), 173.0 (C=O); ES-MS: Calcd for [$C_{33}H_{58}O_{22}N_2S$] 867.6, obs *m/z* 887.1 (M+Na-1), 865.1 (M-1), 432.5 ($M^{2-}$).

*Octyl 2-acetamido-2-deoxy-4-O-[-(α-2,3'-5-acetamido-3,5-dideoxy-D-glycero-α-galacto-non-2-ulopyranosyl)-β-D-galactopyranosyl]-3-O-phosphonato-β-D-glucopyranoside (38)*

$\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.79 (1 H, t, $J_{3"a,e}$ 12.0 and $J_{3"a,4}$ 12.1Hz, 3"-*H*a), 2.03 (6 H, s, 2xNH*Ac*), 2.76 (1 H, dd, $J_{3"a,e}$ and $J_{3"e,4}$ 4.6Hz, 3"-*H*e), 3.5-3.7 (13 H, m), 3.7-4.0 (12 H, m), 4.04 (1 H, dd, $J_{5,6a}$ <2 and $J_{6a,b}$ 11.6Hz, 6a-*H*), 4.1 (1 H, dd, $J_{6a,b}$ and $J_{5,6b}$

3.3Hz, 6b-*H*), 4.21 (1 H, dd, $J_{2,3}$ and $J_{3,4}$ 9.8Hz, 3-*H*), 4.56 (1 H, d, $J_{1,2}$ 8.5Hz, 1-*H*), 4.57 (1 H, d, $J_{1',2'}$ 8.6Hz, 1'-*H*); $\delta_C$ ($D_2O$), 11.5, 20.1 (2xC), 20.6, 23.1, 26.4, 26.6 (2xC), 29.2, 37.8, 49.7, 53.3, 58.2, 59.0, 60.6, 65.6, 66.2, 66.4, 67.2, 68.5, 69.8, 71.0, 73.4 (2xC), 73.7, 97.8, 99.0, 101, 166.7, 171.9, 172.6 (C=O), 173.0 (C=O), 173.9 (C=O); $\delta_P$ ($D_2O$), 0.5.

*Octyl 2-acetamido-2-deoxy-4-O-[-(α-2,3'-5-acetamido-3,5-dideoxy-D-glycero-α-galacto-non-2-ulopyranosyl)-β-D-galactopyranosyl]-6-O-sulfonato-β-D-glucopyranoside (39)*

$\delta_H$ ($D_2O$), 0.6-1.5 (15 H, 3xm, octyl *H*), 1.76 (1 H, t, $J_{3"a,e}$ 12.1 and $J_{3"a,4"}$ 12.2Hz, 3"-*H*a), 1.99 (6 H, s, 2xNH*Ac*), 2.71 (1 H, dd, $J_{3"a,e}$ and $J_{3"e,4"}$ 4.5Hz, 3"-*H*e), 3.4-4.0 (26 H, m), 4.08 (1 H, dd, $J_{5,6a}$ <2 and $J_{6a,b}$ 12.1Hz, 6a-*H*), 4.28 (1 H, dd, $J_{5,6b}$ <2 and $J_{6a,b}$ 6b-*H*), 4.5 (1 H, d, $J_{1,2}$ 7.6Hz, 1-*H*), 4.56 (1 H, d, $J_{1',2'}$ 7.6Hz, 1'-H); $\delta_C$ (D2O), 11.5, 20.1 (2xC), 20.3, 23.1, 26.4, 26.6, 29.2, 37.6, 49.8, 53.2, 59.1, 60.6, 64.4, 65.5, 66.1, 66.5, 67.5, 68.7, 69.5, 70.4, 70.6, 70.9, 73.1, 73.4, 75.3, 97.8, 99.2, 100.2, 172.0 (C=O), 172.4 (C=O), 173.0 (C=O).

*Octyl 2-acetamido-2-deoxy-3-O-(α-D-fucopyranosyl)-4-O-[-(α-2,3'-5-acetamido-3,5-dideoxy-D-glycero-α-galacto-non-2-ulopyranosyl)-β-D-galactopyranosyl]-β-D-glucopyranoside (octyl sialyl Lewis x) (40)*

Sialyl octyl LacNAc (**36**) (2 mg, 2.5 μmol) was dissolved in assay buffer (200 μl, 20 mM HEPES, pH 7 containing 20 mM $MnCl_2$ and 0.2% bovine serum albumin) and the following components were added; GDP-Fuc (1 eq per day for 4 days), enzyme (400 μl, semi-pure human milk α-1,3/4-FucT) and alkaline phosphatase (20 μl, 5U); ES-MS: Calcd for $[C_{39}H_{69}O_{23}N_2]$ 933.5, obs *m/z* 931.9 (M-1), 785.6 (M-Fuc).

*Octyl 2-acetamido-2-deoxy-3-O-(α-D-fucopyranosyl)-4-O-(β-D-galactopyranosyl)-β-D-glucopyranoside (octyl Lewis x) (41)*

Sialyl octyl $Le^x$ (**40**) (1.5 mg, 1.8μmol) was dissolved in HEPES buffer (100 mM, pH 7.0) and neuraminidase type V (isolated from *Clostridium perfringens*, purchased from Sigma) (1U) was added. The assay was incubated at 37 °C for 2 h quenched with water and

purified by standard anion exchange and SepPak chromatography. ES-MS: Calcd for $[C_{28}H_{51}O_{15}N]$ 641.5, obs *m/z* 639.9 (M-1).

*General Procedure for α-1,3-FucT Kinetics Experiments*

Acceptor kinetic assays contained the following components, assay buffer (2 μl, 20 mM HEPES, pH 7 containing 20 mM $MnCl_2$ and 0.2% bovine serum albumin), GDP-Fuc (50 μM, in $H_2O$) and $^3H$ GDP-Fuc[‡] (0.5 μl) enzyme and/or water to 20 μl final volume with 5-6 acceptor concentrations (in duplicate) ranging from 0.2-2 times $K_M$ for each acceptor. After incubations at 37 °C for 10-180 mins, the assays were quenched with water (0.5 $cm^3$) and applied to $C_{18}$ SepPak cartridges previously washed with methanol and equilibrated with water. The cartridges were washed with $H_2O$ (35 $cm^3$) and radiolabelled products eluted with MeOH (4 $cm^3$) directly into scintillation vials. Samples were counted in a Minaxi Tri-Carb 4000 scintillation counter after the addition of Opti-phase Hi-Safe liquid scintillation cocktail (Wallac) (9 $cm^3$).

*General Procedure for β-1,4-GalT Kinetic Experiment*

Acceptor kinetic assays contained the following components, assay buffer (2 μl, 20 mM HEPES, 20 mM $MnCl_2$ containing 0.2% bovine serum albumin), UDP-Gal (10 μM) and $^3H$ UDP-Gal (0.5 μl)[§] enzyme and/or water to 100 μl final volume with 3 acceptor concentrations. After incubations at 37 °C for 30 mins, the assays were quenched with EDTA (850 μl, 10 mM) and applied to $C_{18}$ SepPak cartridges previously washed with methanol and equilibrated with water. The cartridges were washed with $H_2O$ (10 $cm^3$) and radiolabelled products eluted with MeOH (4 $cm^3$) directly into scintillation vials. Samples were counted in a Minaxi Tri-Carb 4000 scintillation counter after the addition of Opti-phase Hi-Safe liquid scintillation cocktail (Wallac) (9 $cm^3$).

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[‡] GDP-Fucose [Fucose-2-$^3$H(N)], 574 GBq/mmol; purchased from NEN

[§] UDP-Galactose UDP-[6-$^3$H]-Gal was purchased from Amersham and had a specific activity of 566 GBq/mmol.

*A Representative Example*

The calculation of the $K_M$ and $V_{max}$ values for the substrate octyl LacNAc 6-sulfate (17) with α-1,3-FucT VI is shown. **Table 5-7** shows the substrate concentrations used and the counts per minute (c.p.m.) obtained from the scintillation counter. A blank assay containing no acceptor gave the c.p.m. for the background (30-90 c.p.m.) and this value was subtracted from the c.p.m. for all other acceptor concentrations.

| **Substrate Concentration (μM)** | **Counts per minute** |
|---|---|
| 5.00 | 2880<br>2674 |
| 2.5 | 2553<br>2629 |
| 1.25 | 2152<br>2072 |
| 0.625 | 1452<br>1457 |
| 0.312 | 906<br>884 |
| 0.156 | 541<br>517 |

**Table 5-7: Raw Data from Octyl LacNAc 6-sulfate $K_M$ Experiment with α-1,3-FucT VI**

A graph of substrate concentration versus rate was plotted using Enzfitter software (version 1.05, published by Elsevier-Biosoft, 1987) and is shown in **Figure 5-8.**

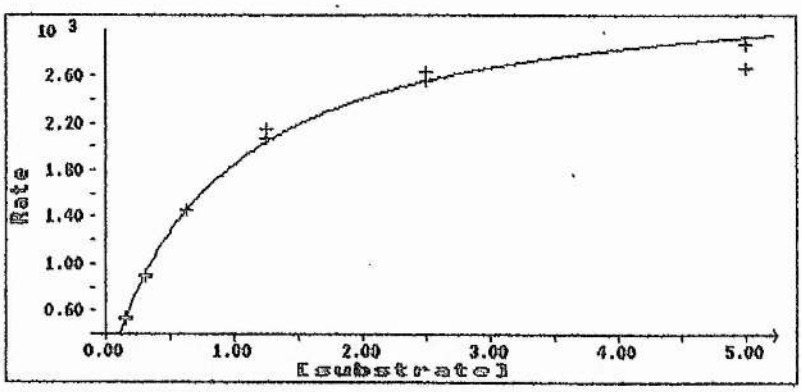



**Figure 5-8: Plot of [S] versus Rate (counts per minute in 30 mins)**

The Lineweaver-Burk double reciprocal plot is shown in **Figure 5-9**.

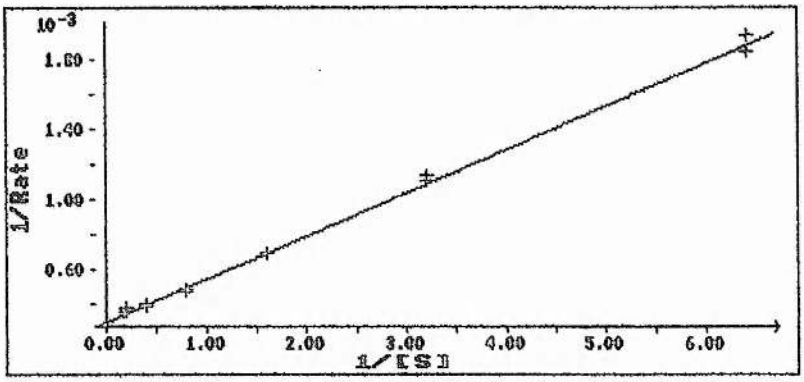



**Figure 5-9: Lineweaver-Burk Plot**

From the non-linear regression plot, the $K_M$ was calculated to be 0.85μM and the $V_{max}$ to be 3940 pmol/min/mg. The errors on calculating these values were 5%. Only the $K_M$ and $V_{max}$ data will be presented for all other calculations. Lineweaver-Burk plots for all experiments are given in **Appendix 2**.

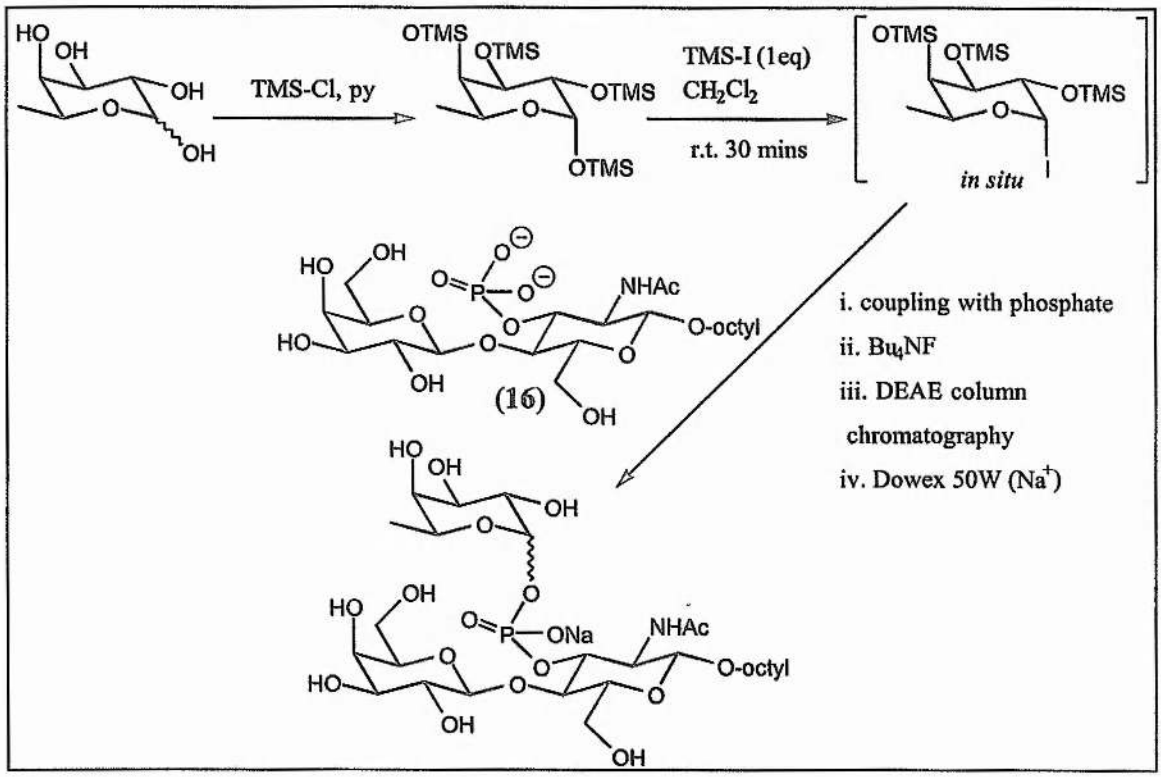
*Procedure for Radiochemical T.L.C. Analysis*

Radiolabelled assay was carried out as described for acceptor kinetic assays. After incubation (3 h) and SepPak purification, the product was eluted from the SepPak with methanol (4 $cm^3$). 10% of the methanol eluent was diluted with scintillation cocktail and counted as described for acceptor kinetic assays. The remainder of the methanol eluent was concentrated to dryness and a known amount of methanol was added. An aliquot of this

sample was loaded onto a t.l.c. plate containing markers of octyl Lewis x (41) and chemically synthesized fucosylated octyl LacNAc phosphate diester (**Figure 5-4**). The t.l.c. was developed ($MeOH/CHCl_3/H_2O$ 10:10:3) and the silica was removed from the plate in 1 $cm^3$ strips. Methanol was added to each silica sample and the suspension was vortexed thoroughly and centrifuged. The supernatant was removed and placed directly into a scintillation vial. This process was repeated three times and the combined MeOH supernatants were diluted with scintillation cocktail and counted as described for acceptor kinetic assays.

*Chemical Synthesis of Fucosylated Octyl LacNAc Phosphate Diester*

The synthesis of the fucose diester linkage to the 3-*O*-phosphate derivative of octyl LacNAc (**16**) was carried out by Taketo Uchiyama in the laboratories of Prof. Ole Hindsgaul at the University of Alberta. **Scheme 5-2** shows the overall synthesis of the desired compound. The general procedure for this reaction can be found in ref. [11].




**Scheme 5-2: Chemical Synthesis of Phosphate Diester Trisaccharide**

*Preparation of n-$Bu_4N$ Salt of (16)*

Na salt of compound (16) (1.3 mg, 0.021 mmol) was dissolved in $H_2O$ (1 $cm^3$) and the solution was passed through Dowex 50W ($H^+$, 200-400 mesh, 0.75 cm x 3 cm) and fractions containing free acid were titrated using aqueous *n*-$Bu_4N(OH)$ (40%) to pH 7. The resulting solution was freeze dried to yield the *tiltle compound* as a white powder.

*Preparation of per-TMS-L-fucopyranose*

To a solution of L-fucose (16 mg, 0.1 mmol) in pyridine (0.5 $cm^3$), chlorotrimethylsilane (76 µl) was added at 0 ° C. After 1 h, *n*-pentane (6 $cm^3$) was added to the reaction mixture which was then extracted with cold $H_2O$ (5x1 $cm^3$). The organic layer was dried over $Na_2SO_4$ and solvent was concentrated to dryness to yield the *title compound* (45 mg, 98%).

*Preparation of fucose-phosphate diester link to octyl LacNAc*

To a solution of per-TMS-L-fuc (9.5 mg, 0.021 mmol) in $CH_2Cl_2$ (0.3 $cm^3$), iodotrimethylsilane (3 µl, 0.021 mmol) was added at r.t. and the reaction mixture was stirred for 30 mins. The reaction mixture was then added to a mixture of (16) (*n*-$Bu_4N$ salt, 0.02 mmol) in dry $CH_2Cl_2$ (2 $cm^3$) and stirred for 3 h. A solution of *n*-$BuN_4F$ (1.0 M, in THF, 63 µl) was added to the reaction mixture and stirred for 1 h. The mixture was concentrated to dryness and the residue was redissolved in $H_2O$ (10 $cm^3$) then applied to DEAE Sephadex (1.5 cm x 12 cm, packed in 20 mM $NH_4HCO_3$) and eluted using a linear gradient from 20 mM to 400 mM $NH_4HCO_3$ (1 $cm^3$ / min, 6 $cm^3$ for each fraction). The appropriate fractions were pooled and concentrated to ~ 10 $cm^3$ (pH=8 to 8.5). Dowex 50W X8 ($H^+$, 200-400 mesh) was then added slowly until neutralisation had occurred. The resin was removed by filtration and the filtrated was passed through a column of Dowex 50W X8 ($Na^+$, 20-50 mesh, 1.5 cm x 5 cm) with water. The eluent was freeze dried as a white powder to give an anomeric mixture of the *title compound* (4 mg, 40%).

## *5.4 References*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# *Chapter 6: Conclusions and Future Work*

## *6.1 Summary and Conclusions*

The target molecules shown in **Figure 6-1** **Figure 6-2** were synthesized in reasonable to high yields. The sialylated compounds (**Figure 6-3**) were prepared using a chemo-enzymatic method using *trans*-sialidase (*T. cruzi*).

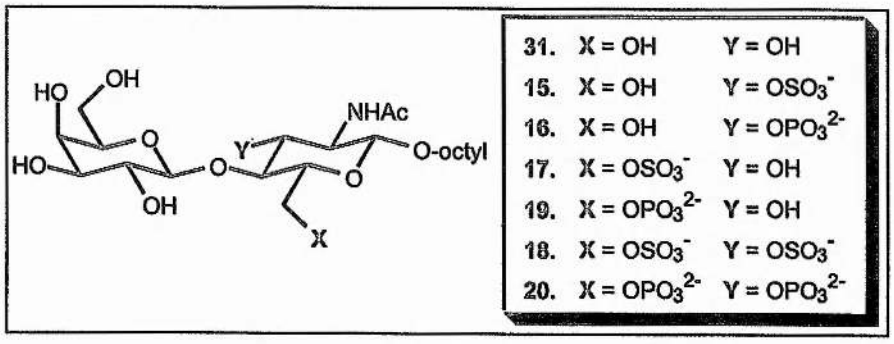


**Figure 6-1: Octyl LacNAc Derivatives**

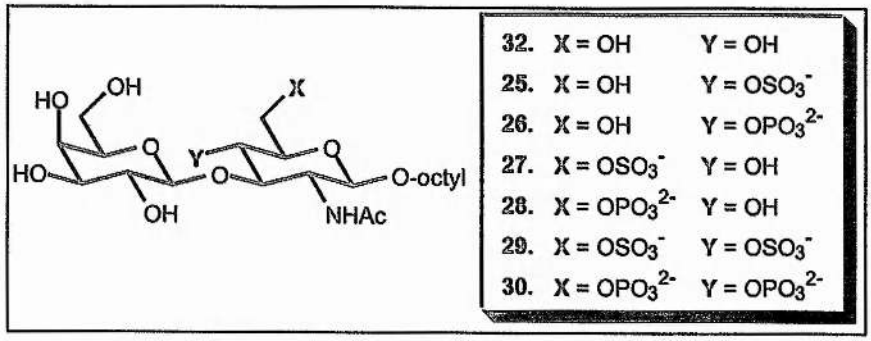


**Figure 6-2: Octyl Gal-β-1,3-GlcNAc Derivatives**

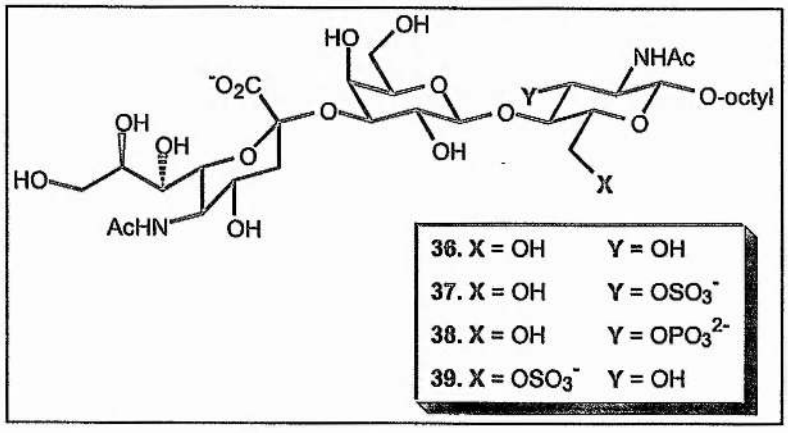


**Figure 6-3: Sialylated Octyl LacNAc Derivatives**

These molecules were screened as potential acceptor substrates for five recombinant α-1,3-FucT's and semi-pure Lewis α-1,3/4-FucT (human milk). It is clear from **Tables 5-1 - 5-6** (**Chapter 5**) that the measured $V_{max}$ values vary dramatically from enzyme to enzyme. Similar observations have been made about recombinant α-1,3-FucTs by deVries and co-workers [1]. It is conceivable that this arises due to the presence of only partially active enzyme as a result of, for instance, misfolding of some of the recombinant enzymes. It is possible, of course, that the various α-1,3-FucTs simply have very different substrate specificities and that we have been looking at inappropriate substrates with some of the enzymes. The relative $V_{max}/K_M$ data quoted can, however, be used to identify trends in the acceptor substrate specificities of various α-1,3-FucTs. **Table 6-1** shows relative $V_{max}/K_M$ data for all the α-1,3-FucT enzymes and all the substrates.

The results shown for α-1,3-FucT V conflict with previously reported data. Murray and co-workers [2] reported Gal-β-1,4-GlcNAc-*O*R (where R= methoxycarbonyloct-1-yl) to have a $K_M$ of 170 μM for recombinant α-1,3-FucT V. However, for the similar substrate Gal-β-1,4-GlcNAc-*O*R (where R= octyl) we found the $K_M$ to be greater than 7 mM and therefore could not be calculated accurately due to problems of insolubility of the substrate. This data does agree, however, with the published work of deVries and co-workers [1] whom reported Gal-β-1,4-GlcNAc-*O*R (where R= methoxycarbonyloct-1-yl) to have a $K_M$ of >10 mM.

| Enzyme/ Substrate | III | IV | V | VI | VII | milk enzyme |
|---|---|---|---|---|---|---|
| **Type I** | | | | | | |
| Gal-β-1,3-GlcNAc-*O*R (32) | 100 | ND | ND | - | ND | 85 |
| 4-*O*-sulfate (25) | - | ND | ND | - | ND | 5 |
| 4-*O*-phosphate (26) | - | ND | ND | - | ND | 2 |
| 6-*O*-sulfate (27) | 46 | ND | ND | - | ND | ND |
| 6-*O*-phosphate (28) | 144 | ND | ND | - | ND | ND |
| **Type II** | | | | | | |
| Gal-β-1,4-GlcNAc-*O*R (31) | - | 100 | - | 100 | 100 | 100 |
| 3-*O*-sulfate (15) | - | - | - | 46 | - | 11 |
| 3-*O*-phosphate (16) | - | - | - | 7 | - | 9 |
| 6-*O*-sulfate (17) | 2 | 202 | 84 | 2126 | - | ND |
| 6-*O*-phosphate (19) | 11 | 115 | 37 | 2245 | - | ND |
| 3,6-di-*O*-sulfate (18) | - | - | - | 11 | - | ND |
| 3,6-di-*O*-phosphate (20) | - | - | - | 8 | - | ND |
| **Type II sialylated** | | | | | | |
| Gal-β-1,4-GlcNAc-*O*R (36) | - | - | 100 | 555 | 14 | ND |
| 3-*O*-sulfate (37) | - | - | 7 | 30 | 58 | ND |
| 3-*O*-phosphate (38) | - | - | - | 6 | - | ND |
| 6-*O*-sulfate (39) | 1 | - | 108 | 1150 | 888 | ND |

**Table 6-1: Relative $V_{max}/K_M$ Data for all α-1,3-FucTs Tested**
R= $-(CH_2)_7CH_3$ (octyl), ND= not determined

An interesting observation was the decrease in $K_M$, relative to the parent unsubstituted compound, measured for compounds containing a sulfate group at the 6-position of octyl LacNAc (17) or octyl Gal-β-1,3-GlcNAc (27). This enhancement in binding affinity of the 6-*O*-sulfate derivatives was observed across all the α-1,3-FucT's examined with the exception of α-1,3-FucT IV (which had $K_M$ similar to the unsubstituted compound). According to Palcic and co-workers [3] the 6-OH was thought to be orientated away from the protein into free solution. If this was the case then the presence of a sulfate

group at the 6-position would be expected to have little or no effect on the binding ability of the substrate. 6-Sulfo $Le^x$ is a natural product [4], so perhaps the 6-*O*-sulfate is simply more related to the true substrate for α-1,3-FucT. This argument would require only one α-1,3-FucT to be affected by the substitution of a sulfate group at the 6-OH. From the results obtained the binding and turnover of the 6-*O*-sulfate with α-1,3-FucT VI has a much enhanced effect, so perhaps α-1,3-FucT VI is involved in the biosynthesis of 6-sulfo Lewis x [4]. However, an effect on binding was observed for most of the α-1,3-FucT's examined and this effect was also observed for type I chains (6-*O*-sulfonato Gal-β-1,3-(α-1,4-Fuc)-GlcNAc is not a reported natural product). A reason for this may be that the presence of an anionic group increases the solubility of the substrates allowing better availability of the substrate in the enzyme active site.

For enzymes tolerating a 3'-sialic acid moiety, a similar pattern was observed with the 6-*O*-sulfate sialylated derivative (39), although the decrease in $K_M$ was not as dramatic as for the non-sialylated substrates. This may be due to the presence of too great an overall charge on the molecules which may result in a tight solvation of the molecule creating steric bulk and thus preventing tight binding of the substrate to the enzyme active site.

In agreement with the unpublished work of Palcic and co-workers, it was also observed that for α-1,3/4-FucT (milk) and α-1,3-FucT VI the 3-*O*-sulfate (15) and 3-*O*-phosphate (16) compounds did act as acceptor substrates with $K_M$ and $V_{max}$ values similar to those of the parent compounds. For α-1,3-FucT V, VI and VII, the sialylated compound containing a sulfate group at position 3 (37) were also observed to be substrates for the enzymes with similar kinetic parameters to sialyl octyl LacNAc (31). As this is the site of glycosylation for α-1,3-FucT, the assumed product must be a sulfate or phosphate diester linkage to fucose (**Figure 6-4**).

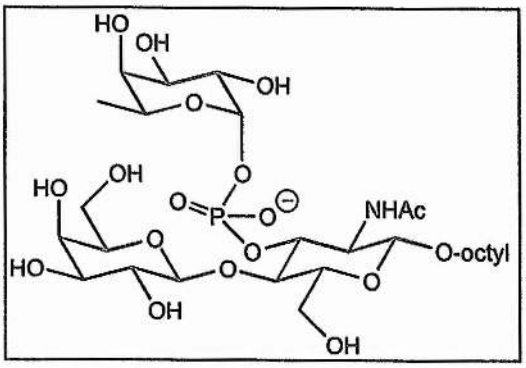


**Figure 6-4: Fucosylated Phosphate Diester Product**

Experiments were carried out to identify the product of the fucosylation reaction. Preliminary results show that the product of the reaction may be the chemically stable phosphate diester shown in **Figure 6-4**. The ability for some α-1,3-FucT's to fucosylate a phosphate group suggests that the model proposed by Lowary and Hindsgaul [5] involving a general base activation of the hydroxyl group to be glycosylated by the enzyme (**Figure 6-5**) does not tell the whole story.

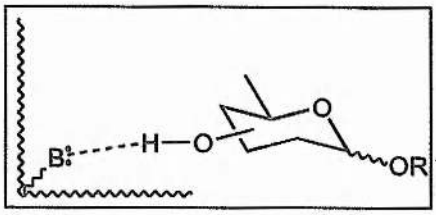


**Figure 6-5: General Base Mechanism for Activation of Hydroxyl Group to be Glycosylated [5]**

However, these results would hold for a model where the manganese cation is involved in binding the hydroxyl group to be glycosylated (**Figure 6-6**) as proposed in **Chapter 1**. The coordination of the manganese cation may have the effect of reducing the pKa of the hydroxyl group of the acceptor substrate and thus increase the nucleophilicity of the oxygen to attack the donor. In the case where the hydroxyl group to be glycosylated of the acceptor molecule is replaced by an anionic group such as a sulfate or phosphate, coordination to the $Mn^{2+}$ should still occur and thus will still be an effective substrate for α-1,3-FucT.

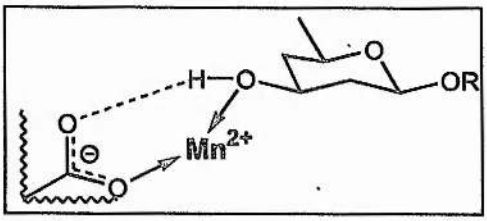


**Figure 6-6: Model Proposed for Activation of Hydroxyl Group to be Glycosylated by $Mn^{2+}$**

## *6.2 Future Work*

### 6.2.1 Phosphate Diester Verification

The work carried out during this study gave preliminary results suggesting that the product of the reaction with octyl LacNAc 3-*O*-phosphate and α-1,3-FucT VI was a fucosylated octyl LacNAc phosphate diester derivative. However in order to confirm the structure of this product it would be necessary to characterize the product by n.m.r. spectroscopy. There should be a distinct change in the $\delta_P$ shift between a phosphate monoester and a phosphate diester and a change in $\delta_H$ shift should also be observed for the H-1 signal of fucose. The assay used to attempt this biotransformation used alkaline phosphatase to degrade the excess GDP forming in the reaction. However this resulted in cleavage of the phosphate from the acceptor substrate. To synthesize sufficient phosphate diester product enzymatically, therefore, will require a new assay system with a different method for removing excess GDP forming during the reaction. A simple solution to this would be to quench, work up and repeat the assay daily to prevent build up of GDP.

### 6.2.2 Verification for the Proposed Model Involving $Mn^{2+}$ Coordination to the Acceptor Substrate

In an attempt to verify the proposed model for $Mn^{2+}$ coordination shown in **Figure 6-6** at least two approaches need to be adopted. The first approach would involve synthesis of thiol and thio-phosphate substrates as these substrates should have a greater affinity with $Mn^{2+}$ than the unsubstituted or sulfated and phosphorylated compounds. The second approach would be to alter the metal cation, replacing $Mn^{2+}$ with $Mg^{2+}$, $Ca^{2+}$and $Co^{2+}$. Full kinetics with all the substrates (sulfate, phosphate, thiol and thiophosphates) and all the metal cations may give support to the proposed model for coordination of the metal cation. If the metal cation ***is*** involved in coordinating the acceptor hydroxyl being glycosylated, then the sulfur containing compounds should be good substrates with thiophilic metals, for example $Mn^{2+}$. Compounds containing sulfate, phosphate or a free hydroxyl ought to have a greater affinity for metals such as $Mg^{2+}$. α-1,3-FucT VI was the only enzyme for which the

3-*O*-sulfate and phosphate derivatives were good substrates. Perhaps the model proposed can only be applied to this particular enzyme. Therefore, it would be important to carry out all experiments with all α-1,3-FucT enzymes to identify whether the metal cation is binding to the hydroxyl group being glycosylated or to an alternative site on the acceptor molecule. This in turn could prove useful in the development of tissue-specific α-1,3-FucT inhibitors for therapeutic uses.

It would also be appropriate to conduct further studies with α-1,3-FucT VI as this appears to be the most active enzyme we have access to and also has the lowest $K_M$ observed for any substrate with α-1,3-FucT. Assuming therefore that $K_M = k_s$ this is a good starting point for making photoaffinity labelled substrates which bind tightly to the enzyme and will yield information about the binding site of the enzyme.

## *6.3 References*


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